EFFECTS OF GROWTH FACTORS AND MEDIA ON THE *EX VIVO* EXPANSION OF CORD BLOOD HEMATOPOIETIC STEM AND PROGENITOR CELLS FOR TRANSPLANTATION

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Abbreviations

7-AAD	7-amino-actinomycin D
ANOVA	analysis of variance
β-ME	β-mercaptoethanol
ACD-A	acid citrate dextrose formula A
BFU/CFU-E	burst-forming unit/colony-forming unit-erythroid
BM	bone marrow
BMT	bone marrow transplantation
BSA	bovine serum albumin
CAFC	cobblestone area-forming cell
CB	cord blood
CFU	colony-forming unit
CFU-GM	colony-forming unit-granulocyte/macrophage
CFU-GEMM	colony-forming unit-granulocyte/erythroid/macrophage/megakaryocyte
CFU-MK	colony-forming unit-megakaryocyte
cy5	cyanine-5
DMSO	dimethyl sulfoxide
EPO	erythropoietin
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FL	flt-3 ligand
FSC	forward scatter
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte/macrophage-colony stimulating factor

GVHD	graft-versus-host disease
HLA	human leukocyte antigen
HPP-CFC	high proliferative potential-colony forming cells
hr	hour
HSA	human serum albumin
IL-1β	interleukin-1β
IL-11	interleukin-11
IL-3	interleukin-3
IL-6	interleukin-6
IMDM	Iscove's Modified Dubecco's Medium
LTC-IC	long-term culture-initiating cells
MBL	mannose-binding lectin
MHC	major histocompatibility complex
min	minutes
MNC	mononuclear cells
NOD/SCID	non-obese diabetic/severe combined immunodeficient
PB	peripheral blood
PBS	phosphate-buffered saline
PBSC	peripheral blood stem cells
PC5	phycoerythrin-cyanine 5-succinimidylester
PCR	polymerase-chain reaction
PE	phycoerythrin
PEG-MGDF	pegylated-megakaryocyte growth and development factor
PI	propidium iodide
rhSCF	recombinant human stem cell factor

rhTPO	recombinant human thrombopoietin
SCF	stem cell factor
SE	standard error
SRC	SCID-repopulating cells
SSC	side scatter
TNC	total nucleated cells
TPO	thrombopoietin

Abstract of thesis entitled:

Effects of Growth Factors and Media on the *Ex Vivo* Expansion of Cord Blood Hematopoietic Stem and Progenitor Cells for Transplantation

Submitted by LAM Audrey Carmen

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at The Chinese University of Hong Kong in June 2001

Human umbilical cord blood (CB) has become an alternative source of hematopoietic stem cells for allogeneic transplantation. However, the varied and small volume in a CB collection often results in insufficient cell doses for either speedy reconstitution of the patient's blood system or engrafting a patient of large body weight. A delayed engraftment may lead to life-threatening infections and bleeding problems. This project was aimed to establish the conditions for the *ex vivo* expansion of enriched CB $CD34^+$ cells in the presence of cytokines, in the attempt to increase the dose of stem and progenitor cells suitable for transplant. Three studies were conducted: (1) comparison of two early-acting cytokines, flt-3 ligand (FL) and stem cell factor (SCF) on the expansion of the megakaryocytic lineage; (2) the effects of a novel factor, mannose-binding lectin (MBL) on the *ex vivo* expansion and preservation of early stem and progenitor cells; and (3) the culture conditions in terms of duration, medium, cytokine combination and autologous plasma for the preclinical *ex vivo* expansion of CB. The read-out system included cell viability, $CD34^+$ and $CD34^+CD38^-$ cells, colony-forming units of the myeloid (CFU-GM), erythroid (BFU/CFU-E), megakaryocytic (CFU-MK) and mixed lineages (CFU-GEMM). The homing and engraftment of expanded human $CD45^+$ cells and subsets were assessed in the bone marrow, spleen and peripheral blood of sub-lethally irradiated non-obese

diabetic (NOD)/severe combined immunodeficient (SCID) mice.

Our results demonstrated that FL was superior to SCF for the expansion of total nucleated cells, $CD34^+$ and $CD34^+CD38^-$ cells, and megakaryocytic progenitors (CFU-MK and $CD61^+CD41^+$ cells). We also demonstrated that flt-3 receptor was not expressed in megakaryocytic cell lines which suggested that the mechanism of FL on the megakaryocytic lineage might be mediated at the stage of hematopoietic progenitor cells.

MBL did not exert any effect on the *ex vivo* expansion of total nucleated cells or progenitor cells of the myeloid and megakaryocytic lineages. However, it increased the yields of early progenitor cells ($CD34^+CD38^-$ cells and CFU-GEMM), particularly in prolonged cultures. More significantly, MBL preserved early stem cells (CFU-GEMM) in serum-free, cytokine-free medium for up to 35 days of culture.

To establish a clinically applicable protocol for the *ex vivo* expansion of CB $CD34^+$ cells, we demonstrated that both serum-free media QBSF-60 and to a lesser extent StemSpan supported the expansion of $CD34^+$ cells. QBSF-60 supported the expansion of $CD34^+$ cells up to 64.8-fold, $CD34^+CD38^-$ cells to 330-fold, CFU-GEMM to 248-fold, CFU-GM to 407-fold and BFU/CFU-E to 144-fold. The expansion of the megakaryocytic lineage was superior in the X-Vivo 10 media (CFU-MK up to 684-fold after 12 days of culture). The addition of autologous CB plasma promoted CFU-GM and BFU/CFU-E but reduced $CD34^+CD38^-$ cells and CFU-GEMM. The addition of granulocyte-colony stimulating factor (G-CSF) or

interleukin-6 (IL-6) to thrombopoietin (TPO), SCF and FL significantly improved the expansion. Expanded cells cultured in QBSF-60 with TPO, SCF and G-CSF engrafted and differentiated into multiple lineages of hematopoietic cells in NOD/SCID mice. Our data supported the strategy of expanding early and myeloid stem and progenitor cells in QBSF-60 and the megakaryocytic lineage in X-Vivo-10 for 12 days in the presence of TPO, SCF, FL and G-CSF without supplementing with FCS or autologous CB plasma. The optimized conditions might be applicable to clinical expansion for the abrogation or reduction of cytopenia in post-transplant patients.

中文摘要：

生長因子和培養基對臍血造血幹/祖細胞體外擴增的影響

人臍血(CB)已用於造血幹細胞移植，但對體重較大的病人，由於 CB 細胞量不足，病人血液系統重建緩慢，導致感染和出血疾病，病人或有生命危險。本研究的目的在於建立體外擴增 CB $CD34^+$ 細胞的條件以增加造血幹/祖細胞的數量。我們進行了三方面的研究： (1) 比較 flt-3 配基 (FL) 和幹細胞因子 (SCF) 對巨核系細胞的擴增； (2) 研究與甘露糖結合的凝集素 (MBL) 對幹/祖細胞體外擴增的影響; (3) CB 體外擴增的臨床前研究。檢測系統包括細胞活性、 $CD34^+$ 和 $CD34^+CD38^-$ 細胞、髓系(CFU-GM)、紅系 (BFU/CFU-E)、巨核系(CFU-MK)、多系 (CFU-GEMM) 集落形成單位和擴增後的細胞在 NOD/SCID 老鼠體內的歸巢和植入能力。

結果表明，FL 擴增有核細胞、$CD34^+$ 和 $CD34^+CD38^-$ 細胞以及巨核祖細胞 (CFU-MK 和 $CD61^+CD41^+$ 細胞)優於 SCF 。巨核細胞系不表達 flt-3 受體，提示 FL 通過介導造血祖細胞來起作用。

MBL 對有核細胞、髓系和巨核系祖細胞的擴增沒有作用，但有利於 $CD34^+CD38^-$ 細胞和 CFU-GEMM 的擴增, 尤其是在長達 35 天的無血清、無細胞因子培養中，MBL 可保存 CFU-GEMM。

研究臨床擴增 CB $CD34^+$ 細胞的條件表明，無血清培養基 QBSF-60 優於 StemSpan 和 X-Vivo 10，可支持 $CD34^+$ 細胞擴增達 64.8 倍、$CD34^+CD38^-$ 細胞達 330 倍、CFU-GEMM 達 248 倍、 CFU-GM 達 407 倍、BFU/CFU-E 達 144 倍。自體血漿促進 CFU-GM 和 BFU/CFU-E 的擴增，但降低了 $CD34^+CD38^-$ 細胞和 CFU-GEMM 的擴增倍數。 添加 G-CSF 或 IL-6 到 TPO、SCF 和 FL 中擴增能力顯著增強。 在 QBSF-60 中添加 TPO、SCF 和 G-CSF，加或不加 FL 培養的細胞可植入 NOD/SCID 老鼠，並可分化爲多系造血細胞。這一優化的培養條件將可用於臨床擴增以減輕移植後的血細胞減少症。

CHAPTER ONE

INTRODUCTION

Section 1.1 Hematopoietic Stem Cells

1.1.1 Hematopoiesis

Hematopoiesis is the formation and development of hematopoietic cells (Bell *et al.* 1997). During embryonic development, the initial appearance of hematopoietic cells was detected at the third week of human gestation in the extraembryonic yolk sac blood islands (Bloom *et al.* 1940, Zon 1995). Recent studies reported the presence of $CD34^+$ hematopoietic cells in the aorta-gonad-mesonephros region of 5-week old embryos, representing the first intraembryonic site of blood development (Peault 1996, Tavian *et al.* 1996, Xu *et al.* 1998, Marshall *et al.* 1999). By the second month of embryonic life, hematopoiesis was transferred to the liver and later supplemented by the spleen, thymus and lymph nodes (Bell *et al.* 1997). The liver remained active in blood cell formation until birth but at the fourth month of gestation, the bone marrow (BM) started to play an increasing role in the synthesis of stem and progenitor cells. Hematopoiesis resided throughout the adult life in the BM. At birth, the umbilical cord blood (CB) was rich in early stem and progenitor cells whereas levels of these cells were low in the normal adult circulation (Christensen *et al.* 1986, Geissler *et al.* 1986, Broxmeyer *et al.* 1989).

1.1.2 Hematopoietic Stem and Progenitor Cells

Pluripotent hematopoietic stem cells have the capacity for self-renewal and differentiation into the complete blood system consisting of erythroid, lymphoid, myeloid and megakaryocytic cells (Figure 1.1). These cells were able to reconstitute the BM in both animal models and human after myeloablative treatments (Bell *et al.*

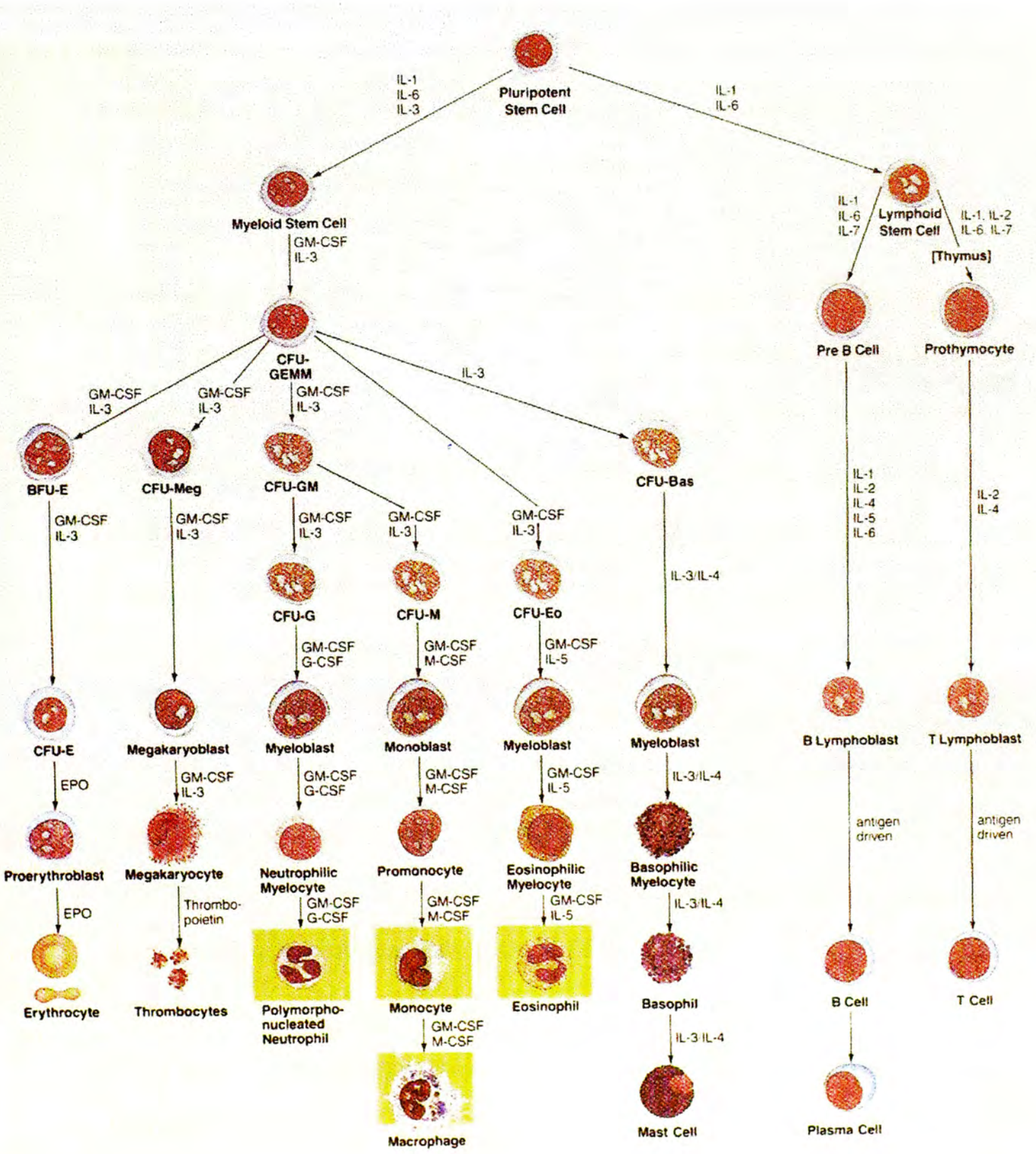


Figure 1.1 Regulation of Hematopoiesis by Cytokines

Cytokines regulate blood cell development by mediating proliferation, differentiation and maturation of hematopoietic progenitor cells. (Reprinted from Bell *et al.* 1997)

1997). Progenitor cells, however, were unable to self-renew and irreversibly committed to one specific cell lineage. In general, it has been suggested that pluripotent stem cells supported long-term engraftment whereas progenitor cells enable short-term engraftment after transplantation.

Hematopoietic stem and progenitor cells have been characterized by the expression of various cell surface markers. The CD34 antigen has been established as the marker for these cells (Civin *et al.* 1984, Berenson *et al.* 1988, Sutherland *et al.* 1989, DiGiusto *et al.* 1994, Galy *et al.* 1994, Bhatia *et al.* 1997a). There has been no single exclusive marker for the identification of pluripotent stem cells. However, the subpopulations of $CD34^+$ cells which were lineage marker negative, such as $CD38^-$ and HLA-DR^-, were considered to contain the early progenitor cells (Sutherland *et al.* 1989, Verfaillie *et al.* 1990, Terstappen *et al.* 1991, Verfaillie *et al.* 1993, Huang *et al.* 1994). Recently, Ziegler *et al.* (1999) reported that the vascular endothelial growth factor receptor 2 or KDR might be a marker for pluripotent stem cells. KDR^+ cells were about 0.1 – 0.5% of human $CD34^+$ cells. In addition, it has been suggested that the early quiescent stem cells might be $CD34^-$ (Osawa *et al.* 1996, Goodell *et al.* 1997, Bhatia *et al.* 1998, Zanjani *et al.* 1998, Goodell 1999, Sato *et al.* 1999) and the CD34 antigen expression might be up-regulated in response to proliferative signals (Goodell 1999).

For functional assays of hematopoietic progenitors, *in vitro* assays of colony-forming units (CFU), long-term culture-initiating cells (LTC-IC), cobblestone area-forming cells (CAFC) and the *in vivo* non-obese diabetic (NOD)/severe combined

immunodeficient (SCID) mouse transplantation model have been used for the identification of early stem and progenitor cells of various specific lineages.

Section 1.2 Stem Cell Transplantation

1.2.1 Stem Cell Transplantation

Hematopoietic stem cell transplantation has been proven to provide definitive therapy for a variety of high-risk or recurrent hematologic malignancies, marrow failure syndromes, selected hereditary immunodeficiency states, metabolic disorders and solid tumors. Hematopoietic stem cells could be obtained from related or unrelated donors (allogeneic transplant). Alternatively, stem cells might be collected from cancer patients (autologous transplant) at remission and these cells could be reinfused to them prior to subsequent high-dose chemotherapy/radiotherapy. However, the infusion of autologous stem cells might result in the re-introduction of contaminating cancer cells (Takvorian *et al.* 1987).

1.2.2 Sources of Hematopoietic Stem Cells for Transplantation

Since the first successful bone marrow transplantation (BMT) was carried out almost 40 years ago (Thomas *et al.* 1959), BM has been the source of donor stem and progenitor cells. The establishment of human leukocyte antigen (HLA) typing enabled the selection of major histocompatibility complex (MHC)-compatible donor/recipient pairs (Dausset *et al.* 1970, Bach *et al.* 1976). The main limitations to allogeneic BMT have been the lack of suitable HLA-matched donors and the complications of graft-versus-host disease (GVHD) associated with HLA disparities.

Peripheral blood stem cells (PBSC) have been used as a source of hematopoietic stem and progenitor cells for transplantation since 1990s (Rizzo 1998). The normal adult peripheral blood (PB) did not contain significant amount of stem and progenitor cells. Upon stimulation by cytokines such as granulocyte-colony stimulating factor (G-CSF) (Stahel *et al.* 1994, Goldman 1995, Klumpp *et al.* 1995, Lane *et al.* 1995, Li *et al.* 1999) and granulocyte/macrophage-colony stimulating factor (GM-CSF) (Corringham *et al.* 1995, Kessinger *et al.* 1995, Lane *et al.* 1995, Passos-Coelho *et al.* 1995), hematopoietic stem and progenitor cells could be mobilized into PB from the BM and collected by leukapheresis. The leukapheresis procedure was considered to be less morbid than BM harvest by most patients. However, the long-term side effects of the mobilizing agents have not been assessed.

Allogeneic and autologous PBSC transplants have been performed for the treatment of malignant and non-malignant diseases (Brugger *et al.* 1995, Alcorn *et al.* 1996, Williams *et al.* 1996, Bertonlini *et al.* 1997a, Holyoake *et al.* 1997, Reiffers *et al.* 1999, McNiece *et al.* 1999, McNiece *et al.* 2000a, Paquette *et al.* 2000). When PBSC transplantation was compared with BMT, rapid neutrophil and platelet recoveries were observed in patients received PBSC (Bensinger *et al.* 1996, Urbano-Ispizua *et al.* 1996, Champlin *et al.* 2000). Champlin *et al.* (2000) reported that significantly shorter duration of neutrophil (median 14 days) and platelet (18 days) recovery were observed in 288 patients receiving PBSC from their HLA-identical siblings when compared to the neutrophil (19 days) and platelet (25 days) recovery of 536 BMT patients.

1.2.3 Cord Blood as a Source of Hematopoietic Stem Cells

The feasibility of using human umbilical CB as a source of transplantable stem and progenitor cells for hematopoietic reconstitution was suggested by Broxmeyer *et al.* (1989). The first allogeneic CB transplant was performed successfully in 1988 for treating a child with Fanconi's anemia (Gluckman *et al.* 1989). The donor of this CB was his HLA-identical sister. The patient showed full hematopoietic and lymphoid reconstitution. As a result, CB banks have been established worldwide to facilitate the supply of CB for allogeneic transplants (Rubinstein *et al.* 1993, Rubinstein *et al.* 1995, Kurtzberg *et al.* 1996, Gluckman *et al.* 1997, Guardiola *et al.* 2000). At present, over 30,000 CB units have been in storage and about 1,500 CB transplants have been performed (Kurtzberg *et al.* 1996, Wagner *et al.* 1995, Gluckman *et al.* 1997, Rubinstein *et al.* 1998, Gluckman 2000).

1.2.3.1 Advantages of Cord Blood Transplant

Umbilical CB has numerous advantages over BM and PBSC as a source of stem and progenitor cells for transplantation. These included the ease and safety of collection, lower incidence of infectious agents such as cytomegalovirus (Sirchia *et al.* 1999). CB could be fully tested and HLA-typed prior to cryopreservation and would be readily available upon request for transplantation.

One major advantage of CB transplant has been the reduced incidence and severity of acute GVHD (Vormoor *et al.* 1994, Gluckman *et al.* 1997, Rubinstein *et al.* 1998, Gluckman 2000). Analyzing 138 CB transplants in related children, Gluckman (2000) reported that the probabilities of developing acute GVHD $\geq$ II and chronic GVHD were 20% and 6%, respectively. In 291 unrelated children and 108 adult

transplants, the acute GVHD ≥ II were 39% and 38%, respectively. These figures compared favorably with unrelated BMT of which the acute GVHD ≥ II was 58% (Gluckman 2000). In general, CB transplants could tolerate 1 – 2 HLA mistmatches between donors and recipients. Therefore, there might be a higher probability of finding a suitable CB unit for transplantation when compared to the location of a BM donor, in spite of the fact that more than 5 millions BM donors have been included in BM donor registries worldwide (Gluckman 2000).

Hematopoietic stem and progenitor cells in CB were considered as high quality and possessed greater proliferative potential than those of BM and PB (Leary *et al.* 1987, Carow *et al.* 1991, Verfaillie 1992, Carow *et al.* 1993, Lu *et al.* 1993, Hirao *et al.* 1994, Vormoor *et al.* 1994, Hao *et al.* 1995, Traycoff *et al.* 1995, Mayani *et al.* 1998, Lewis *et al.* 2000). The quantity of LTC-IC in CB was higher when compared to BM (Hows *et al.* 1992, Traycoff *et al.* 1995) and PB (Hirao *et al.* 1994, Lewis *et al.* 2000). The number of CFU-GEMM has been shown to be 4-fold higher in CB than in BM (Mayani *et al.* 1998). Lu *et al.* (1993) reported that the frequency of high proliferative potential colony-forming cells (HPP-CFC) were 8-fold higher in CB than in BM. Moreover, the frequencies of $CD34^{+}HLA\text{-}DR^{-}$ and $CD34^{+}CD38^{-}$ cells were higher in CB than in BM as reported by Cardoso *et al.* (1993).

1.2.3.2 Disadvantages of Cord Blood Transplant

A major disadvantage of CB transplant has been the once-only collection, varied and relatively low cell number in a CB unit. As a result, most transplants especially those carried out in early years were performed in patients with small body weights. Recently, it has been confirmed that the cell dose in a CB was associated with the

outcomes of engraftment. Gluckman (2000) reported that in adult transplants, none of the patients who received less than 1×10^7 nucleated cells/kg body weight survived. Patients who received less than 2×10^7 nucleated cells/kg had a 69% probability of reaching $\geq$ 500/μl neutrophils and 49% of reaching $\geq$ 20,000/μl platelets on day 60. It was thus recommended that the minimum nucleated cell dose in a CB unit should be 4×10^7/kg of the body weight of the recipient.

In general, the engraftment kinetics of CB transplant was slower when compared to that using other sources of hematopoietic stem and progenitor cells. Gluckman (2000) reported the median durations to neutrophil and platelet engraftments in adult CB transplants were 32 and 81 days, respectively and were significantly higher than those receiving allogeneic BMT. It has been uncertain whether the delays of engraftment were results of low cell doses or due to the prematurity of CB stem and progenitor cells. The delayed reconstitution of the hematopoietic system in transplant patients often resulted in prolonged hospitalization and frequent transfusions of blood cells and platelets. In this period, the patients suffered from severe myelosuppression or even myeloablation and might develop complications including life-threatening infections and bleeding.

Section 1.3 *Ex Vivo* Expansion

The *ex vivo* expansion of hematopoietic stem and progenitor cells under defined conditions in the presence of cytokines has been suggested as an approach for overcoming the inadequate number of these cells in a CB unit (Broxmeyer *et al.* 1992, Cardoso *et al.* 1993, Moore *et al.* 1994, Ruggieri *et al.* 1994, Traycoff *et al.* 1994a, Xiao *et al.* 1994). The *ex vivo* expansion of CB cells was first performed by

Broxmeyer *et al.* (1992) who cultured mononuclear cells (MNC) from CB and BM in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum (FCS) with cytokine cocktails consisted of stem cell factor (SCF), interleukin-3 (IL-3), erythropoietin (EPO), GM-CSF and G-CSF. After 7 days, higher fold increases of CFU were obtained in CB cultures than in BM cultures. Since then, numerous studies have been undertaken to develop the optimal system for the *ex vivo* expansion of hematopoietic stem and progenitor cells for clinical applications (Moore 1995, Emerson 1996, Aglietta *et al.* 1998, Conrad *et al.* 1998, Shih *et al.* 2000, McNiece 2001). These included the selection of the appropriate cell populations for expansion, the use of new reagents, cytokines, procedures for cell culture and the development of approved materials and equipment for clinical expansion.

Various assays have been used to evaluate the culture outcomes, particularly the expansion of primitive hematopoietic progenitor cells. Differential CFU assays were employed to assess the expansion of early and committed progenitor cells of the mixed (CFU-GEMM), myeloid (CFU-GM), erythroid (BFU/CFU-E) and megakaryocytic (CFU-MK) lineages (Alcorn *et al.* 1996, Bertolini *et al.* 1997a, Bachier *et al.* 1999, Kögler *et al.* 1999). Flow cytometry has also been used to distinguish between various progenitor cell subpopulations such as $CD34^+$ cells and $CD34^+CD38^-$, $CD34^+CD61^+$ cells (Alcorn *et al.* 1996, Bertolini *et al.* 1997a, Kögler *et al.* 1999, Paquette *et al.* 2000). Concurrently, *in vivo* animal transplantation techniques such as the NOD/SCID mouse (Wermann *et al.* 1996, Bhatia *et al.* 1997b, Bhatia *et al.* 1998, Guenechea *et al.* 1999, Piacibello *et al.* 1999) and sheep (Yin *et al.* 1997, Almeida-Porada *et al.* 2000) models were developed for the assessment of the expansion products.

1.3.1 Optimization of Expansion Conditions

1.3.1.1 $CD34^+$ Cell Selection

The CD34 antigen is a single-chain 115 kDa cell surface transmembrane glycophosphoprotein. At least three distinct CD34 epitopes have been identified (Lansdorp *et al.* 1989). *In vitro* and *in vivo* data suggested that the human $CD34^+$ cell compartment contained primitive multipotential hematopoietic cells for transplantation (Srour *et al.* 1991).

Several studies suggested that $CD34^+$ cell selection was necessary for the optimal expansion of hematopoietic stem and progenitor cells (Laver *et al.* 1995, Zimmerman *et al.* 1995, Charbord *et al.* 1996, Briddell *et al.* 1997, Cornetta *et al.* 1998, Fietz *et al.* 1999). Using selected CB $CD34^+$ cells, Briddell *et al.* (1997) showed that the fold increases of total nucleated cells, CFU-GM and BFU-E were 113, 72.6 and 49.0, respectively after 10 days of cultures, whereas the expansions were only 1.4-, 0.8- and 0.3-fold, respectively in cultures using unselected CB cells. On the other hand, a continuously perfused culture system (CPS) has been developed by Aastrom Biosciences (Koller *et al.* 1993) who expanded MNC from CB, BM and PBSC without $CD34^+$ cell enrichment (Zandstra *et al.* 1994, Koller *et al.* 1995, Sandstrom *et al.* 1995, Koller *et al.* 1998). Recently, this system has been applied for clinical expansion (Bachier *et al.* 1999, Pecora *et al.* 2000, Stiff *et al.* 2000).

A number of methods have been used for the preclinical and clinical enrichment of $CD34^+$ cells. In some protocols, $CD34^+$ cell separation was effected by the collection of antibody-sensitized cells onto a solid phase such as magnetic beads or columns of non-magnetic particles (Chang *et al.* 1985, Ugelstad *et al.* 1986, Holyoake *et al.*

1997, Kögler *et al.* 1999). Commercially developed systems for $CD34^+$ cell selection included the Isolex 300 (Nexell; Irvine, CA, USA), Ceprate SC (CellPro Inc., Bothell, WA, USA) and Magnetic Activation Cell Sorting (MACS) System (Miltenyi Biotec. Inc.; Gladbach, Germany) (Thomas *et al.* 1994, Korbling *et al.* 1994, Miltenyi *et al.* 1994). For research purposes, high-speed flow cytometry has also been employed for $CD34^+$ cell sorting (Uchida *et al.* 1994). Alternatively, CD34 negative selection could be performed (StemSep, Stem Cell Technologies, Vancouver, BC, Canada) (Bertolini *et al.* 1997b).

1.3.1.2 Cytokines

Hematopoiesis is largely governed by a number of cytokines that promote the renewal, proliferation, and differentiation of hematopoietic stem and progenitor cells (Figure 1.1). To date, more than 50 cytokines have been shown to play some roles in the regulation of hematopoiesis (Al-Homsi *et al.* 2000). Cytokines were secreted by a wide variety of cells, including mesenchymal cells such as fibroblasts and endothelial cells, monocytes, granulocytes, mast cells, T and B lymphocytes (Gualtieri *et al.* 1984, Crosier *et al.* 1992). Non-hematopoietic organs such as the liver and kidneys also produced specific cytokines (Crosier *et al.* 1992).

Cytokines are hormone-like peptides. They exerted their biological function by binding to specific receptors on target cells. These receptors have been classified into two major families, the hematopoietic receptors and tyrosine kinase receptors (Olsson *et al.* 1992, Kishimoto *et al.* 1994). The hematopoietic receptor family included receptors for EPO, GM-CSF, G-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7 and IL-9. The family of tyrosine kinase receptors included receptors for thrombopoietin

(TPO), SCF, flt-3 ligand (FL) and colony-stimulating factor-1 (CSF-1). However, IL-1 and IL-8 receptors did not belong to any of the two families.

Some cytokines shared common signal transduction pathway. Cytokines regulated hematopoiesis by mechanisms such as modulation of cell cycling, potentiation of expression of receptors of other growth factors and regulation of apoptosis (Tanaka *et al.* 1995). A number of cytokines have been demonstrated to promote *ex vivo* expansion of hematopoietic stem and progenitor cells. These included TPO, SCF, FL, G-CSF, GM-CSF, IL-1β, IL-3, IL6, IL-11, EPO, macrophage inflammatory protein-1α (MIP-1α), promegapoietin (a chimeric IL-3/TPO receptor agonist). (Naparstek *et al.* 1992, Brugger *et al.* 1995, Alcorn *et al.* 1996, Williams *et al.* 1996, Bertolini *et al.* 1997a, Holyoake *et al.* 1997, Kögler *et al.* 1999, Bachier *et al.* 1999, Reiffers *et al.* 1999, Kratz-Albers *et al.* 2000, McNiece *et al.* 1999, Pecora *et al.* 2000, Stiff *et al.* 2000, Paquette *et al.* 2000, McNiece *et al.* 2000a).

1.3.1.2.1 Thrombopoietin

TPO has been cloned (Barnett *et al.* 1994, Kaushansky *et al.* 1994, Lok *et al.* 1994, Wendling *et al.* 1994) and considered to be the primary regulator of megakaryocytopoiesis (de Sauvage *et al.* 1994, Kuter *et al.* 1994). The TPO receptor (*c-mpl*) was first reported in the murine myeloproliferative leukemia virus as the transforming oncogene known as *v-mpl* (Wendling *et al.* 1986). It was identified on megakaryocytes, platelets and primitive hematopoietic progenitors (Vigon *et al.* 1992, Young *et al.* 1996, Ramsfjell *et al.* 1996). Mice lacking either TPO or its receptor were severely thrombocytopenic (Gurney *et al.* 1994, Alexander *et al.* 1996, de Sauvage *et al.* 1996). These mice also had significant deficiencies in both early

and lineage-committed progenitor cells in the BM (Alexander *et al.* 1996, Carver-Moore *et al.* 1996, Kimura *et al.* 1998). In addition, administration of TPO to normal or myelosuppressive mice resulted in the expansion of all lineages of progenitors in their BM and spleen (Han *et al.* 1995, Farese *et al.* 1996).

TPO acted on various levels of megakaryocytopoiesis by stimulating the formation of megakaryocyte colonies, maturation of immature megakaryocytic progenitors, increase in megakaryocyte ploidy and formation of functional platelets (de Sauvage *et al.* 1994, Lok *et al.* 1994, Broudy *et al.* 1995, Kaushansky 1995, Cramer *et al.* 1997). In *ex vivo* cultures, TPO alone markedly increased the commitment of stem and progenitor cells to the megakaryocytic lineage but did not significantly enhance their proliferation (Sieff *et al.* 1989, Briddell *et al.* 1990, Briddell *et al.* 1992, Quesenberry *et al.* 1991, van den Oudenijn *et al.* 1999, Su *et al.* 2001). However, TPO enhanced the expansion of megakaryocytic progenitors in the presence of other cytokines (Cortes *et al.* 1995, Guerriero *et al.* 1995, Choi *et al.* 1996, Birkmann *et al.* 1997, Borge *et al.* 1997, Dolzhanskiy *et al.* 1997, Gehling *et al.* 1997, Ramsfjell *et al.* 1997, Williams *et al.* 1998, van den Oudenijn *et al.* 1999, Su *et al.* 2001). TPO also acted synergistically with other early-acting cytokines such as SCF and FL in supporting the expansion of early progenitor cell populations including CFU-GEMM and $CD34^{+}CD38^{-}$ cells (Ziegler *et al.* 1994, Ramsfjell *et al.* 1996, Kobayashi *et al.* 1996, Liu *et al.* 1999).

A homolog of TPO, the recombinant human pegylated-megakaryocyte growth and development factor (MGDF) has been developed for clinical applications to patients suffering from thrombocytopenia. The administration of TPO to cancer patients receiving chemotherapy resulted in dose-dependent increases of platelet counts and

BM megakaryocytes (Basser *et al.* 1996, O'Malley *et al.* 1996). However, the use of MGDF has been challenged due to reports on the development of neutralizing antibodies in some patients and normal volunteers (Bolwell *et al.* 2000, Fields *et al.* 2000, Neumann *et al.* 2000).

1.3.1.2.2 Stem Cell Factor

SCF has been demonstrated to play a pivotal role in the regulation of early hematopoiesis by binding to its receptor c-kit, which was widely expressed in human hematopoietic cells (Flanagan *et al.* 1990, Huang *et al.* 1990, Williams *et al.* 1990a, Zsebo *et al.* 1990, André *et al.* 1989, Wang *et al.* 1989, Hu *et al.* 1994, Morita *et al.* 1996). Mice with dominant mutations at the SCF and c-kit genes displayed a substantial reduction in the number of hematopoietic stem cells with phenotypes such as hypoplastic anemia and mast cell deficiency (Broudy 1997).

As an early-acting cytokine, SCF has been extensively used in the *ex vivo* expansion of human $CD34^+$ cells (Haylock *et al.* 1992, Srour *et al.* 1992, Henschler *et al.* 1994, Mayani *et al.* 1994, Moore *et al.* 1994, Williams *et al.* 1996). SCF increased all lineages of CFU including CFU-GM, BFU-E, CFU-GEMM and CFU-MK (Brandt *et al.* 1992, Shapiro *et al.* 1996, Lyman 1998). Ohmizono *et al.* (1997) reported that the combination of SCF with TPO, IL-3 and IL-6 or IL-11 enhanced the expansions of CB $CD34^+$ cells to CFU-GM, BFU-E and CFU-GEMM after 14 days of cultures. The addition of SCF to cultures of BM $CD34^+$ cells increased the expansion of $CD34^+$ cells by 0.96 to 2.64-fold (Luens *et al.* 1998).

SCF was reported to enhance the effects of G-CSF in the mobilization of PBSC in the mouse model (Briddell *et al.* 1993, Andrews *et al.* 1994a, Yan *et al.* 1994, Yan *et*

al. 1995) and in human clinical trials (Andrews *et al.* 1994b, Glaspy 1996). In the latter, SCF, in addition to filgrastim, increased the peak peripheral blood $CD34^+$ cell level by 2-3 fold (Glaspy *et al.* 1997, Weaver *et al.* 1998).

1.3.1.2.3 Flt-3 Ligand

FL has been shown to possess hematopoietic, lymphopoietic and immunologic activities (Lyman 1998). The flt-3 receptors were widely expressed in tissues of brain, thymus, skin, kidney, liver and lymph node (Rosnet *et al.* 1991, Meierhoff *et al.* 1995). In the hematopoietic system, flt-3 receptors were identified in stem and progenitor cells, granulocytic-monocytic cells, B-lymphocytes and acute leukemic cells of lymphoid and myeloid origins (Birg *et al.* 1992, Small *et al.* 1994, Brasel *et al.* 1995, Meierhoff *et al.* 1995). Mice deficient in flt-3 had impaired primitive hematopoietic progenitor cell functions and reduced B lymphocyte precursors in their BM (Mackarehtschian *et al.* 1995). Repeated administration of FL in mice increased the hematopoietic progenitor cells in the BM and differentiation of these cells into the lymphoid, myeloid and erythroid lineages (Brasel *et al.* 1996). In addition, the *in vivo* administration of FL induced functional dendritic cells in mice (Maraskovsky *et al.* 1996).

For the *ex vivo* expansion of BM or CB $CD34^+$ cells, FL alone had minimal effects on the formation of CFU. It produced a small number of CFU-GM, but no BFU-E nor CFU-GEMM. However, in combination with other cytokines, such as SCF, IL-3 and GM-CSF, FL enhanced the expansion of both early and committed progenitor cells (CFU-GM, BFU-E, CFU-GEMM and HPP-CFC) (Broxmeyer *et al.*1995). In the analysis of the effects of 16 cytokines on $CD34^+CD38^-$ cells, Petzer *et al.* (1996) reported that FL was the only cytokine to increase the number of LTC-IC from BM

$CD34^+CD38^-$ cells by 30-fold after 10 days of culture. Piacibello *et al.* (1996a) suggested that T- and B- lymphocytes could also be expanded in the presence of FL. When BM $CD34^+$ cells were expanded with FL, IL-7 and IL-11 for 14 days, the percentages of $CD2^+$ and $CD19^+$ cells reached 33.8% and 3.7%, respectively. In the NOD/SCID mouse transplantation model, cells expanded with cytokine cocktails containing FL were able to engraft and repopulate in the BM of recipient mice (Conneally *et al.* 1997, Dorrell *et al.* 2000, Kusadasi *et al.* 2000, Ueda *et al.* 2000, Yamaguchi *et al.* 2001). FL increased the engraftment ability of expanded cells in NOD/SCID mice by 10 – 13.8 fold (Kusadasi *et al.* 2000).

FL has been applied to mobilize PBSC in non-human primates together with G-CSF and resulted in the significant increase of CFU in their PB. (Papayannopoulou *et al.* 1997). Administration of FL to patients with metastatic colon cancer led to an increase in the number of circulating dentritic cells (Morse *et al.* 2000).

1.3.1.2.4 Granulocyte-Colony Stimulating Factor

G-CSF has been known to promote the myeloid lineage. The G-CSF receptor was expressed on hematopoietic cells including pluripotent and myeloid-committed progenitors, neutrophils, monocytes and possibly certain lymphocyte subsets (Demetri *et al.* 1991). It was also expressed on endothelial cells and G-CSF induced their proliferation *in vitro* (Bocchietto *et al.* 1993).

G-CSF acted synergistically with other cytokines on cell proliferation (Metcalf *et al.* 1995). It increased the neutrophil count and shortened the duration of neutropenia in patients after cytotoxic treatments (Demetri *et al.* 1992, Glaspy *et al.* 1992). G-CSF

appeared to be the most effective single agent for the mobilization of neutrophils and $CD34^+$ cells for transplantation purposes (Stahel *et al.* 1994, Goldman *et al.* 1995, Klumpp *et al.* 1995, Lane *et al.* 1995, Li *et al.* 1999). Recently, G-CSF has been included in several preclinical and clinical studies on the *ex vivo* expansion of hematopoietic stem and progenitor cells (Briddell *et al.* 1997, Andrews *et al.* 1999, Kögler *et al.* 1999, McNiece *et al.* 1999, Reiffers *et al.* 1999, Kobari *et al.* 2000, Kratz-Albers *et al.* 2000, McNiece *et al.* 2000a, McNiece *et al.* 2000b, Paquette *et al.* 2000).

1.3.1.2.5 Interleukin-3

IL-3 has been considered as a multipotent hematopoietic growth factor produced by activated T cells, monocytes/macrophages and stromal cells (Mangi *et al.* 1999). It also promoted early megakaryocytopoiesis (Emerson *et al.* 1988). As a single factor, IL-3 supported the proliferation of hematopoietic progenitor cells after they exited from the cell cycle G_0 state (Suda *et al.* 1985, Ogawa 1993). It also synergized with TPO (Kobayashi *et al.* 1996, Ku *et al.* 1996), SCF (Tsuji *et al.* 1991, Tsuji *et al.* 1992), G-CSF (Ikebuchi *et al.* 1988), IL-6 (Ikebuchi *et al.* 1987) and IL-11 (Musashi *et al.* 1991a, Musashi *et al.* 1991b) in triggering cell divisions of the multipotential progenitors in G_0. Addition of IL-3 to TPO with IL-1, IL-6, IL-11 and SCF resulted in a 1.6-fold increase in the expansion of total nucleated cells (van den Oudenrijn *et al.* 1999). Bryder *et al.* (2000) demonstrated that IL-3 supported the expansion of multipotential progenitors ($Lin^-Sca\text{-}1^+Kit^+$ cells) derived from mouse BM cells in the presence of TPO, SCF and FL.

However, the effects of IL-3 on the maintenance or promotion of early repopulating cells during expansion have been controversial (Knobel *et al.* 1994, Yonemura *et al.* 1995, Shimizu *et al.* 1998). Some studies reported that IL-3 expanded cells engrafted in NOD/SCID mice (Bhatia *et al.* 1997b and Conneally *et al.* 1997). However, Knobel *et al.* (1994) and Yonemura *et al.* (1995) demonstrated that IL-3 abrogated the reconstituting ability of hematopoietic stem cells *in vivo*. Piacibello *et al.* (2000) demonstrated that IL-3 exhibited an inhibitory effect on the expansion of human long-term repopulating hematopoietic stem cells. This observation was in agreement with those of Peters *et al.* (1996) and Matsunaga *et al.* (1998), who demonstrated that the addition of IL-3 to BM cultures in the presence of IL-6, IL-11 and SCF resulted in the impairment of the engrafting capability in NOD/SCID mice.

Various clinical trials of IL-3 have been performed for the development of new therapeutic strategies for cancer patients (Mangi *et al.* 1999). Administration of IL-3 to patients with relapsed lymphomas, small-cell lung cancer, breast cancer and ovarian cancer resulted in the reduction of chemotherapy delays and the faster regeneration of granulocytes and platelets (Dercksen *et al.* 1993, Pedrazzoli *et al.* 1994). IL-3 has been applied in combination with GM-CSF or G-CSF for PBSC mobilization and produced higher cell yields (Dercksen *et al.* 1995). However, the treatment of myelodysplastic syndromes, aplastic anemia and other BM disorders with IL-3 has not been effective (Kudoh *et al.* 1996).

1.3.1.2.6 Interleukin-6

IL-6 has been reported to promote early hematopoiesis (Ikebuchi *et al.* 1987, Jacobsen *et al.* 1994) and megakaryocytopoiesis (Ishibashi *et al.* 1989, Lotem *et al.*

1989, Imai *et al.* 1991). A complex of IL-6 and soluble IL-6 receptor (IL-6/sIL-6R) enhanced the expansion of multipotential hematopoietic progenitor cells through gp130 signaling (Ebihara *et al.* 1997, Sui *et al.* 1999). Peters *et al.* (1997) showed that IL-6/sIL-6R double transgenic mice had extramedullary expansion of hematopoietic progenitor cells and a substantial increase of circulating platelets and other lineages of hematopoietic cells.

IL-6 acted in synergy with other factor on the proliferation of multipotential hematopoietic progenitor cells (Piacibello *et al.* 1999, Rappold *et al.* 1999, Moore 2000). The combination of IL-6, FL and TPO supported long-term expansion of total nucleated cells, $CD34^+$ cells and CFU-GM (Lazzari *et al.* 2001a). The addition of IL-6 to cultures of CB $CD34^+$ cells with TPO, SCF and FL improved the engraftment of expanded cells in NOD/SCID mice (Kusadasi *et al.* 2000). For the megakaryocytic lineage, IL-6 stimulated the late developmental stages of megakaryocyte maturation and polyploidization (Ishibashi *et al.* 1989, Navarro *et al.* 1991, Teramura *et al.* 1992, Broudy *et al.* 1995).

Phase I and II trials of IL-6 treatments have been performed in patients with solid tumours, aplastic anaemia and B-lymphoproliferative disorders (Weber *et al.* 1993, Banks *et al.* 1995, Schrezenmeier *et al.* 1995, Bouffet *et al.* 1997, Bracho *et al.* 2001, Haddad *et al.* 2001). However, high incidence of toxicity was observed in these patients, possibly related to the induction of proinflammatory mediators (Schrezenmeier *et al.* 1995, Bracho *et al.* 2001).

1.3.1.2.7 Comparison of Flt-3 Ligand and Stem Cell Factor

FL was structurally related to SCF (Broxmeyer *et al.* 1991, Roth *et al.* 1992). Lyman *et al.* (1995) demonstrated that while SCF stimulated the expansion of both $CD34^+$ and $CD34^+CD38^-$ cells, FL only promoted the latter population. The strong synergism between FL and SCF suggested that these cytokines were not functionally redundant (Lyman *et al.* 1998) and that FL might have a distinct contribution to early hematopoietic development.

FL and SCF also promoted megakaryocytopoiesis. They enhanced the proliferation of megakaryocytic progenitors, but had little effect on mature megakaryocytes (Briddell *et al.* 1991, Broudy *et al.* 1995, Piacibello *et al.* 1996a). The mechanism of these two early cytokines on the expansion of the megakaryocytic lineage has not been clear. Some studies reported that FL promoted better expansion of megakaryocytic progenitors than SCF (Bertonlini *et al.* 1997b, Ohmizono *et al.* 1997, Shapiro *et al.* 1996). However, others demonstrated that SCF was more efficient for the expansion of this lineage (Williams *et al.* 1998). In addition, FL could amplify the megakaryocytic proliferative effects of SCF (Piacibello *et al.* 1996b).

1.3.1.2 Culture Medium

IMDM has been extensively used in the expansion of hematopoietic stem and progenitor cells. FCS, human serum albumin (HSA) or autologous plasma have also been included as nutrient supplements. The use of serum-free culture medium has been proposed for clinical applications in order to minimize the risk of viral or other infections, variations between individual batches of serum and regulatory issues.

Table 1.1 Comparison of Serum and Serum-free Medium on the *Ex Vivo* Expansion

Cell Source	Cytokines	Media Compared	Recommended Medium	Reference
BM	SCF, IL-1, IL-3	DMEM + FCS, Progenitor-34	Progenitor-34	Lebkowski *et al.* 1994
CB and PB	SCF, IL-3, IL-6	IMDM + FCS, HLTM, AIM V, Progenitor-34, QBSF-59	QBSF-59	Sandstrom *et al.* 1996
PB	SCF, EPO, IL-1β, IL-3, IL-6	IMDM + serum substitutes, RPMI + FCS, ex vivo 10, ex vivo 20, QB58, QB59	IMDM + serum substitutes	Möbest *et al.* 1998
CB	SCF, FL, EPO, GM-CSF, IL-3	RPMI + FCS, H5100	H5100	Kögler *et al.* 1998
CB and PB	SCF, FL, G-CSF, GM-CSF, EPO, IL-3, IL-6	HLTM, X-Vivo 20	X-Vivo 20	Collins *et al.* 1998
CB	SCF, FL, IL-3, IL-6, MIP-1α	IMDM + FCS, StemPro-34	StemPro-34	Capmany *et al.* 1999
PB	SCF, G-CSF, IL-3, IL-6	IMDM + FCS / autologous plasma, X-Vivo 10	IMDM + autologous plasma	Mellado-Damas *et al.* 1999
BM	TPO, IL-3	IMDM + human serum, Easymega	Easymega	Lefebvre *et al.* 1999
PB	TPO, IL-3	IMDM, CellGro, StemSpan	StemSpan	van den Oudenrijn *et al.* 2001
CB	TPO, FL, IL-6, IL-11	StemPro, CellGro, X-Vivo 10	StemPro, CellGro	Lazzari *et al.* 2001

Capmany *et al.* (1999) demonstrated that expansion systems using a specific serum-free medium resulted in more consistent outcomes when compared to serum-containing cultures. Several commercially available serum-free media have been developed and were shown to support more efficient expansion of hematopoietic stem and progenitor cells when compared to those using IMDM and FCS (Lebkowski *et al.* 1994, Sandstrom *et al.* 1996, Collins *et al.* 1998, Kögler *et al.* 1998, Möbest *et al.* 1998, Capmany *et al.* 1999, Lefebvre *et al.* 1999, Mellado-Damas *et al.* 1999, Lazzari *et al.* 2001b, van den Oudenrijn *et al.* 2001) (Table 1.1).

1.3.2 Mannose-Binding Lectin

Lectins are glycoproteins which bind to specific carbohydrates in a reversible manner. They are ubiquitous in nature and present in microorganisms, plants and animals. They have been suggested to play a role in cancer, fertilization, immune response and signal transduction. Mannose-binding lectins are those that bind specifically to mannose (Meier *et al.* 2000).

In human, MBL has been isolated in human serum and was involved in innate immunity (Saifuddin *et al.* 2000). In the presence of calcium, MBL could bind to the repeating sugar arrays on microbial surfaces, leading to the direct uptake by phagocytes. Low serum levels of MBL were observed in some patients with suspected immunodeficiencies, unexplained infections and systemic lupus erythematosus (Turner 1998). MBL was suggested to be an inhibitor of human immunodeficiency virus type-1 and cytomegalovirus replication *in vitro* (Balzarini *et al.* 1991, Balzarini *et al.* 1992).

Plant MBL have been shown to exert some effects on hematopoietic cells. The *in vivo* treatment with MBL extracted from bulbs (Ooi *et al.* 1998) induced the expression of IL-1β, TNF-α and immuno-reactive nitric oxide synthetase in splenocytes and macrophages of mice (Ooi *et al.* 2001). Recently, the Flt-3 receptor-interacting lectin (FRIL) extracted from red kidney beans stimulated the proliferation of NIH 3T3 cells transfected with flt-3 (Colucci *et al.* 1999). They also reported that FRIL possessed the ability to preserve hematopoietic progenitors in long-term cultures (29 days) without exogenous support of serum or growth factors. In the *ex vivo* expansion system, incubation of $CD34^+$ cells with FRIL resulted in lower numbers of cycling cells compared with cytokine-stimulated cells and these cultured cells successfully engrafted the NOD/SCID mice (Kollet *et al.* 2000).

1.3.3 *Ex Vivo* Expansion for Clinical Transplantation

The first attempt of expanding hematopoietic cells for clinical transplantation was performed by Naparstek *et al.* (1992) who cultured one-third of allogeneic BM cells with GM-CSF and IL-3 for 4 days. These cells, together with the uncultured BM cells were infused into 20 patients who were suffering from malignant hematological diseases. There was no significant acceleration of neutrophil recovery in these patients but a shorter median platelet recovery time was observed when compared to historical controls. Subsequently, several clinical trials were performed and the details of the expansion systems and outcomes were summarized in Table1.2.

Brugger *et al.* (1995) described the first report on the clinical transplant using expanded autologous PBSC without simultaneous infusion of uncultured cells. He demonstrated that no toxic effect or side effect was associated with the infusion of

expanded cells. The neutrophil and platelet recovery times were 13 and 14 days, respectively and were not different from those of historical controls. However, there have been concerns that the chemotherapy regimen employed was not myeloablative. A similar study was performed by Alcorn *et al.* (1996) who expanded $CD34^+$ cells enriched from cryopreserved PB in serum-free Progenitor-34 medium containing 5 – 10% autologous serum. Ten patients with nonmyeloid malignancy were infused with expanded cells and showed rapid neutrophil and platelet engraftments (10 and 12 days). However, Holyoake *et al.* (1997) reported that 2 non-Hodgkin's lymphoma and 2 multiple myeloma patients who received expanded cells using the same cytokine combination failed to show durable engraftment.

Thrombocytopenia was a significant cause of morbidity in patients undergoing high-dose chemotherapy and hematopoietic stem cell transplantation (Kessinger *et al.* 1988, Williams *et al.* 1990b). Regular platelet transfusions were costly and associated with the risk of infectious diseases, alloantibody formation and subsequent refractoriness to the transfusion. The rate of platelet recovery correlated with the number of infused megakaryocytic progenitors (Leibundgut *et al.* 1995, Takamatsu *et al.* 1995, Feng *et al.* 1998). Using a protocol for the expansion of megakaryocytic lineage, Bertonlini *et al.* (1997a) reported that two out of four patients receiving the highest numbers of infused megakaryocytic progenitors did not require platelet transfusion. The median duration of neutrophil and platelet engraftment was 9 and 11 days, respectively.

The *ex vivo* expansion of CB stem and progenitor cells has become a promising strategy to overcome the limitation of the small cell dose in a CB unit. Kögler *et al.*

(1999) first reported the expansion of CB $CD34^+$ cells with TPO, FL, G-CSF. Expanded cells as well as unmanipulated $CD34^+$ cells were infused into a patient who suffered from acute lymphoblastic leukaemia. The neutrophil and platelet recovery times were 14 and 75 days, respectively. Subsequently, several trials were performed on the clinical expansion of CB hematopoietic progenitor cells (Kurtzberg *et al.* 1999, Goldberg e*t al.* 2000, Pecora e*t al.* 2000, Shpall *et al.* 2000) and favorable results were observed in some studies (Pecora *et al.* 2000, Shpall *et al.* 2000).

Instead of using culture flasks or large-scale culture bags, a perfusion culture system was developed by Aastrom Bioscience for clinical BM and CB cell expansion (Koller *et al.* 1993, Koller *et al.* 1995, Koller *et al.* 1998, Bachier *et al.* 1999, Kurtzberg *et al.* 1999, Goldberg *et al.* 2000, Pecora *et al.* 2000, Stiff *et al.* 2000). In this system, MNC were expanded in FCS-containing long-term culture medium with PIXY321 (a fusion protein of GM-CSF and IL-3), FL and EPO for 12 days. Most of patients receiving only expanded cells or simultaneously with uncultured cells experienced sustained neutrophil and platelet engraftments. No toxicity or side effect was observed in them except those reported by Goldbery *et al.* (1999). In this trial using expanded CB cells, 12 out of 19 patients died due to GVHD, sepsis, adenovirus infection and central nervous system hemorrphage.

In other trials, the medium and cytokines (TPO, SCF and G-CSF) of Amgen Inc. (Thousand Oaks, CA, USA) were used for the clinical expansion of PBSC and CB. Some favorable outcomes were reported (Reiffers *et al.* 1999, McNiece *et al.* 1999, Paquette *et al.* 2000, McNiece *et al.* 2000). Reiffers *et al.* (1999) demonstrated that

Table 1.2 ***Ex Vivo*** **Expansion for Clinical Transplantation**

Cell Source	Patients	Cytokines	Culture Medium	Culture Duration (days)	Engraftment Outcome compared with Historical Controls	Remarks	Reference
BM	20 malignant hematological diseases	GM-CSF, IL-3	RPMI + human AB serum	4	faster platelet recovery and shorter hospitalization period, 4 out of 20 died (2 GVHD and 2 other causes)	infused with 2/3 uncultured cells with 1/3 cultured cells	Naparstek *et al.* 1992
PB	10 advanced cancers	SCF, IL-1β, IL-3, IL-6, EPO	RPMI + autologous plasma	12	no difference in neutrophil and platelet recovery, 1 died of multiorgan failure	4 patients infused with uncultured and expanded $CD34^+$ cells	Brugger *et al.* 1995*
PB	10 nonmyeloid malignancy	SCF, IL-1β, IL-3, IL-6, EPO	Progenitor-34 + autologous serum	8	no difference in neutrophil and platelet recovery	all patients received only a single infusion of expanded cells	Alcorn *et al.* 1996*
PB	8 breast cancer	PIXY321	X-Vivo 10 + HSA	12	prompt neutrophil (8 – 10 days) and platelet (8 – 12 days) recovery	infused with uncultured and expanded cells	Williams *et al.* 1996*
PB	8 breast cancer, 2 non-Hodgkin's lymphoma	TPO, SCF, FL, IL-3, IL-6, IL-11, MIP-1α	X-Vivo 10	7	2 of 4 patients receiving the highest doses did not need platelet transfusion	2 patients infused with uncultured and expanded cells	Bertolini *et al.* 1997*
PB	2 non-Hodgkin's lymphoma, 2 multiple myeloma	SCF, IL-1β, IL-3, IL-6, EPO	Progenitor-34 + autologous serum	8	all failed to show neutrophil and platelet engraftment	unmanipulated cells were infused as back-up	Holyoake *et al.* 1997
CB	1 acute lymphoblastic lymphoma	TPO, FL, G-CSF	X-Vivo 10 + autologous CB plasma	10	complete and stable hematopoietic and lymphopoietic engraftment	infused both uncultured and expanded cells	Kögler *et al.* 1999*

Cell Source	Patients	Cytokines	Culture Medium	Culture Duration (days)	Engraftment Outcome	Remarks	Reference
BM	5 breast cancer	PIXY321, FL, EPO	Long-term culture medium	12	all sustained neutrophil and platelet engraftment	Aastrom CPS infused both uncultured and expanded cells	Bachier *et al.* 1999*
PB	14 multiple myeloma	TPO, SCF, G-CSF	Amgen-defined serum-free medium	10	significantly shorter neutrophil (0 – 7 days) and platelet (0 – 5 days) recovery	infused both uncultured and expanded cells	Reiffers *et al.* 1999*
PB	12 breast cancer	TPO, SCF, G-CSF	Amgen-defined serum-free medium	10	prompt neutrophil recovery, no effects on platelet recovery	2 received unmanipulated $CD34^+$ cells	McNiece *et al.* 1999*
CB	28	PIXY321, EPO, FL	Long-term culture medium	12	no difference in time to myeloid, erythroid and platelet engraftment	Aastrom CPS infused both uncultured and expanded cells	Kurtzberg *et al.* 1999*
CB	19 myeloid leukemia, multiple myeloma, non-Hodgkin's lymphoma	PIXY321, FL, EPO	Long-term culture medium	12	12 toxic death, 3 relapse, 1 infection death	Aastrom CPS	Goldberg *et al.* 2000
CB	37 leukemia and breast cancer	TPO, SCF, G-CSF	Amgen-defined serum-free medium	10	shorter neutrophil and platelet recovery	infused both uncultured and expanded cells	Shpall *et al.* 2000*

Cell Source	Patients	Cytokines	Culture Medium	Culture Duration (days)	Engraftment Outcome	Remarks	Reference
CB	2 chronic myelogenous leukemia	PIXY321, FL, EPO	Long-term culture medium	12	sustained neutrophil and platelet engraftment	Aastrom CPS infused both uncultured and expanded cells	Pecora *et al.* 2000*
BM	19 breast cancer	PIXY321, FL, EPO	Long-term culture medium	12	similar neutrophil recovery, 1 patient had delayed platelet engraftment	Aastrom CPS	Stiff *et al.* 2000*
PB	24 breast cancer	TPO, SCF, G-CSF	IMDM + HSA	9	reduced neutropenia, thrombocytopenia & anemia	1 had delayed platelet engraftment	Paquette *et al.* 2000*
PB	21 breast cancer	TPO, SCF, G-CSF	Amgen-defined serum-free medium	10	shorter neutrophil recovery, no effects on platelet recovery	2 expansion cohorts	McNiece *et al.* 2001*

* No toxicity or side effect was observed after transplantation.

absolute and severe neutropenia could be abrogated in 14 multiple myeloma patients after high-dose chemotherapy by the infusion of both expanded and uncultured cells. Median duration of severe neutropenia was dramatically reduced to 1.5 days (range 0 – 7 days) when compared to historical records. McNiece *et al.* (1999, 2000) transplanted expanded cells (from PBSC) and uncultured cells to patients suffering from breast cancer and again demonstrated shortened durations of neutrophil engraftment but improvements in platelet recovery were not observed. Paquette *et al.* (2000) cultured unselected PBSC mobilized from 24 breast cancer patients. The patients had shorter times to neutrophil and platelet recovery and fewer red cell transfusions when compared to historical controls. Seven patients had neutropenia for 3 or fewer days and 9 patients did not experience neutropenic fevers. The median time for platelet recovery was 9.3 days.

Section 1.4 Non-Obese Diabetic/Severe Combined Immunodeficient Mouse Transplantation Model

It is critical that the stem and progenitor cells produced after *ex vivo* expansion possess the abilities to "home" to the bone marrow and differentiate into functional blood cells of different lineages. An *in vivo* transplant model would be required the assessment of such functions. Over the past decade, some mouse mutants have been employed for the transplant of human hematopoietic stem and progenitor cells. In 1983, the severe combined immunodeficient (SCID) mouse, which was homozygous for the *Prkdc*^{scid} locus, was developed (Bosma *et al.* 1983). These mice were severely deficient in functional B and T lymphocytes but readily supported xenogeneic tumors and human hematopoietic cells (Bankert *et al.* 1989, McCune *et al.* 1989, Dick 1991). Cesano *et al.* (1992) demonstrated that established cell lines and primary

samples from patients with acute or chronic myeloid leukemia could be reproducibly grown in SCID mice.

In the SCID/hu model developed by McCune *et al.* (1988), a human hematopoietic microenvironment was created by implanting fragments of human fetal thymus and liver (McCune *et al.* 1988) or bone (Kyoizumi *et al.* 1992) into C.B-17-*scid/scid* mice. However, the engraftment levels of human cells were low, representing only 0.5% – 5% of the total recipient marrow population. The murine-origin microenvironment in SCID mice might not be physiologically sufficient to sustain human stem cell self-renewal and differentiation.

Recently, SCID mice have been backcrossed with the non-obese diabetic (NOD/Lt) mouse background. In addition to lacking T- and B-cell functions, the resulting NOD/LtSz-*scid/scid* (NOD/SCID) mice exhibited low natural killer cell activity, defective macrophage function and lacking hemolytic complement (Shultz *et al.* 1995). NOD/SCID mice have shown improved engraftment of human hematopoietic cells over SCID mice (Bock *et al.* 1995, Shultz *et al.* 1995, Larochelle *et al.* 1996, Verlinden *et al.* 1998).

Human cells capable of engrafting in mice were termed SCID-repopulating cells (SRC) (Lapidot *et al.* 1992, Larochelle *et al.* 1996). Larochelle *et al.* (1996) demonstrated that most LTC-IC and CFU were incapable of engrafting NOD/SCID mice, thus providing strong evidence that SRC was more primitive and a distinct cell population. SRC were phenotypically characterized as $CD34^+CD38^-$ cells (Larochelle *et al.* 1996). Recent studies demonstrated that $CD34^+CD38^-$ cells (Bhatia

et al. 1998) and $CD34^+CD38^+$ cells (Conneally *et al.* 1997) have limited engraftment potential. It has been suggested that there might be two distinct classes of SRC, the long-term repopulating cells that had life-long ability to produce all blood cell types and the short-term repopulating cells that were either myeloid or lymphoid restricted progenitors (Jordan *et al.* 1990, Keller *et al.* 1990, Magli *et al.* 1982, Kondo *et al.* 1997, Akashi *et al.* 2000). Hogan *et al.* (1997) firstly reported the characterization of hematopoietic development of human CB $CD34^+$ cells in NOD/SCID mice. They showed that human CB $CD34^+$ cells could consistently engraft in the BM of these mice. Over 90% of recipients who survived to the end of the experiment showed varying degrees of human cell repopulation. $CD34^+$ cells engrafted and proliferated through the hematopoietic tissues such as PB and spleen of NOD/SCID recipients.

Christianson *et al.* (1997) developed a new strain of immunodeficient mice in which the residual low natural killer activity present in the NOD/SCID mouse was essentially eliminated by backcrossing the β2 microglobulin-null ($\beta2m^{-/-}$) allele onto the NOD/SCID background. Both long-term and short-term repopulating cells were able to engraft NOD/SCID-$\beta2m^{-/-}$ mice (Glimm *et al.* 2001), thus allowing the assessment of the most primitive types of human stem cell populations.

CHAPTER TWO

OBJECTIVES

The overall objective of this study was to investigate and establish an optimal culture system for the *ex vivo* expansion of cord blood stem and progenitor cells. The specific aims were:

(1) To investigate the effects of flt-3 ligand and stem cell factor on the *ex vivo* expansion of megakaryocytic progenitor cells and the expression of flt-3 ligand receptors in megakaryocytic cells

The effects of two early-acting cytokines FL and SCF on the expansion of enriched $CD34^+$ cells to megakaryocytic progenitors were assessed in cultures containing TPO, IL-3 and IL-6. The read-out system included viable nucleated cells, $CD34^+$ cells, $CD34^+CD38^-$ cells, $CD61^+CD41^+$ cells and CFU-MK. The expressions of FL receptor were measured on four megakaryocytic cell lines and three leukemic bone marrow samples by flow cytometry analysis.

(2) To study the effects of a novel agent, mannose-binding lectin on the *ex vivo* expansion system and the preservation of primitive progenitors

An in-house developed plant MBL, in increasing dosage, was included in expansion cultures with two cytokine combinations for 14 – 35 days. The read-out system was $CD34^+$ cells and their $CD38^-$ subsets, CFU of the mixed, erythroid, myeloid and MK lineages as well as the NOD/SCID mouse transplantation model. To investigate the possible property of MBL on the preservation of early stem cells, it was included in CB MNC or $CD34^+$ cell cultures in serum-free X-Vivo 10 medium without cytokine supplements for 35 days. The presence of CFU was then determined.

(3) To optimize the culture duration, culture media, addition of autologous plasma and cytokine combinations for the preclinical *ex vivo* expansion of cord blood $CD34^+$ cells

The expansions were performed with various treatments: four media, four cytokine combinations, two culture duration and autologous CB plasma as a supplement.

CHAPTER THREE

MATERIALS AND METHODOLOGY

Section 3.1 Collection of Cord Blood Samples

All CB samples were collected with informed consent and the approval of the Ethics Committee for Clinical Research of The Chinese University of Hong Kong.

CB samples were collected into 30 ml sterile plastic universal containers (Sterilin; Stone, UK) containing preservative-free sodium heparin (David Bull Laboratories; Victoria, Australia) at a final concentration of 10 IU/ml in RPMI 1640 medium (Gibco; Grand Island, NY, USA) supplemented with 100 IU/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco). CB was collected from the umbilical vein after the umbilical cord was clamped during normal vaginal deliveries of full-term newborns. All aspects of cord clamping and placental delivery were at the discretion of midwives and clinicians in the labor ward of Department of Obstetrics and Gynecology, Prince of Wales Hospital.

Section 3.2 Cryopreservation and Thawing of Cord Blood

For some experiments as specified, CB samples were cryopreserved before use. Fresh unmanipulated CB samples were mixed with an equal volume of ice-cold freezing medium containing 40% RPMI 1640 medium, 40% HSA (Baxter; Glendale, CA, USA) and 20% dimethyl sulphoxide (DMSO) (Sigma; St. Louis, MO, USA) in freezing bags (Cryocyte, Nexell; Irvine, CA, USA). These bags were then placed in protective aluminum canisters and subjected to rate-controlled freezing using a controlled rate freezer (CryoMed; New Baltimore, MI, USA). The freezing chamber

was cooled at 5°C per min to 4°C and equilibrated for 10 min. It was then cooled at 2°C per min to –30°C, at 4°C per min to –100°C and equilibrated for 10 min. Subsequently, the bags were transferred into the liquid phase of liquid nitrogen tank for storage.

CB samples were kept in liquid nitrogen for at least 48 hr before use. Thawing of CB was performed as described by Rubinstein *et al.* (1995). CB bags were equilibrated in the gas phase of liquid nitrogen for 30 min and then exposed to ambient temperature for 5 min to allow the plastic to regain elasticity. The bags were immersed in a 37°C water bath immediately and thawed with continuous agitation and gentle massage by hand. Usually it took less than 2 min to thaw a whole blood unit. The CB was then diluted with an equal volume of washing solution containing 5% HSA and 10% Dextran 40 (McGaw; Irvine, CA, USA) in 0.9% normal saline and centrifuged at 400 × g for 10 min at 4°C. The supernatant was removed and the cells were resuspended in fresh washing solution prior to $CD34^+$ cell enrichment.

Section 3.3 Enrichment of $CD34^+$ Cells

MNC were first prepared by density gradient centrifugation over Ficoll Hypaque (1.077 g/ml) (Amersham Pharmacia; Uppsala, Sweden). Fresh CB samples were diluted 1:1 with IMDM (Gibco). Cryopreserved CB samples were thawed, washed and resuspended in washing solution to the volume as originally collected. Thirty ml freshly diluted CB or thawed CB were carefully layered over 15 ml of Ficoll Hypaque and centrifuged without brake at 400 × g for 35 min at room temperature. The upper layer was aspirated leaving the MNC layer undisturbed at the interphase. The interphase cells were collected carefully. The cells were then washed two times

with Ca^{2+} and Mg^{2+} free phosphate-buffered saline, pH 7.4 (PBS) (Gibco) supplemented with 0.6% acid citrate dextrose formula A (ACD-A) (Baxter; Deerfield, IL, USA) and centrifuged at 200 × g for 10 min at room temperature. Cell count was performed using a Bright-light hemacytometer (Reichert-Jung, Cambridge Instruments Inc.; Buffalo, NY, USA). The viability of cells was evaluated by the trypan blue exclusion assay. Cells were diluted 1:4 with 0.1% (w/v) trypan blue (Gibco) and dye-excluded cells were counted with the hemacytometer. The viability was reported as the percentage of viable cells after counting 400 nucleated cells.

$CD34^+$ cells were enriched using the MACS CD34 Progenitor Isolation Kit (Miltenyi Biotec. Inc.; Gladbach, Germany). According to the manufacturer's instruction, MNC were resuspended at 10^8 cells per 300 μl in PBS buffer containing 0.6% ACD-A and 0.5% deionized bovine serum albumin (BSA) (Sigma). All labeling reagents provided in the isolation kit were added at 100 μl per 10^8 total cells. For the processing of less than or more than 10^8 cells, the amount of reagents and antibodies were adjusted accordingly. The maximum concentration of the cell suspension was 10^8 cells per 500 μl. The cells were incubated with a FcR blocking reagent (human IgG) and simultaneously with anti-human monoclonal hapten-conjugated CD34 antibody (Clone QBEND/10) for 15 min at 4°C with shaking at 5 min intervals. After washing with 10 ml PBS buffer, the cells were incubated with colloidal super-paramagnetic MACS Microbeads conjugated to an anti-hapten antibody for 15 min at 4°C with shaking every 5 min. These cells were then washed and resuspended at 10^8 cells per 500 μl PBS buffer.

The positive selection MS^+ or VS^+ column which could process up to 10^8 or 10^9 cells, respectively was used and placed (with column adaptor) in the magnetic field of the Mini or Vario MACS separator. For experiments on the "Effects of flt-3 ligand and stem cell factor on the expansion of megakaryocytic progenitor cells" in Section 3.4.1, MS^+ columns and MiniMACS were used while VS^+ columns and VarioMACS were used in experiments on the "Effects of mannose-binding lectin on the *ex vivo* expansion of hematopoietic stem and progenitor cells" in Section 3.4.2 and "Optimization of culture conditions for the preclinical *ex vivo* expansion of cord blood hematopoietic stem and progenitor cells" in Section 3.4.3. The column was filled and rinsed with 500 µl (for MS^+ column) or 3 ml (for VS^+ column) PBS buffer. The cells were loaded onto the column. After unbound cells have passed through the column, it was washed with 500 µl (for MS^+ column) or 3 ml (for VS^+ column) PBS buffer for three times. The MS^+ or VS^+ column was then removed from the separator and placed on a 6 ml tube (Falcon, Becton Dickinson, BD; Franklin Lakes, NJ, USA) or a 12 ml collection tube (Miltenyi), respectively. Retained cells were eluted using the plunger with 0.5 ml or 2 ml PBS buffer pipetting onto the MS^+ or VS^+ column, respectively. The eluted cells were applied to a second prefilled column. Enriched $CD34^+$ cells were finally obtained by repeating the washing and elution steps.

The purity of isolated $CD34^+$ cells was evaluated by flow cytometry described in Section 3.6 and the viability of cells was determined by the trypan blue exclusion assay.

Section 3.4 *Ex Vivo* Expansion

All culture media were supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. The choice of cytokine combinations was determined by their ability to support the expansion of various lineages of progenitor cells. In Section 3.4.1, megakaryocytic-specific cytokines were used. In Section 3.4.2, two groups of cytokine combinations were employed for the expansion of megakaryocytic and early progenitors. In Section, 3.4.3, cytokines of clinical or preclinical grades were assessed. Concentrations of cytokines used were based on published data and prior dose-response experiments (Luens *et al.* 1998, McNiece *et al.* 1999, Murray *et al.* 1999, Reiffers *et al.* 1999, Sui *et al.* 1999, Lewis *et al.* 2000, McNiece *et al.* 2000b, Paquette *et al.* 2000, Ueda *et al.* 2000, Yang *et al.* 2000, Su *et al.* 2001).

Cytokines used in Section 3.4.1, 3.4.2, 3.5.1 and 3.5.2 were diluted in IMDM plus 1% BSA at a concentration of 1 ng/μl. Cytokines in Section 3.4.3 were diluted in QBSF-60 serum-free medium (Quality Biological; Gaithersburg, MD, USA) plus 1% HSA at a concentration of 5 ng/μl. All cytokines, except those provided by Kirin Brewery Co., Ltd. (Tokyo, Japan), were stored in aliquots at –80°C following manufacturer's recommendations. Cytokines provided by Kirin Brewery Co., Ltd. were kept at 4°C after dilution. MBL used in Section 3.4.2 was developed in-house from a plant source in the Department of Biology, The Chinese University of Hong Kong. Due to possible development of this product for commercial purposes, details of its synthesis and characterization were not disclosed. MBL was diluted in IMDM plus 1% BSA at a concentration of 10 ng/μl and kept at –80°C.

3.4.1 Effects of Flt-3 Ligand and Stem Cell Factor on the Expansion of Megakaryocytic Progenitor Cells

3.4.1.1 Ex Vivo Expansion of Cord Blood $CD34^+$ Cells with Flt-3 Ligand or Stem Cell Factor

Enriched $CD34^+$ cells at 8×10^4 were cultured in a 24-well culture plate (Falcon) in 1 ml IMDM FCS (Gibco) in the presence of 20 ng/ml TPO, IL-3 and IL-6 which were megakaryocytic-specific cytokines plus either 20 ng/ml FL or SCF. All cytokines were products of Peprotech (Rocky Hill, NJ, USA). The cultures were incubated at 37°C and 5% CO_2 in a fully humidified atmosphere for 21 days. At days 7 and 14, three-quarters of cells from each well were harvested for analysis. Equal volumes of fresh medium and cytokines were added. At day 21, all cells were harvested for analysis. Seven independent CB cultures were performed.

At each time point, cell counts, viability, flow cytometry analysis and CFU-MK colony assay were performed. The total nucleated cell count was calculated by multiplying the number of nucleated cells per ml by the volume of cells. After counting the number of cells on the eight quadrants of the hemacytometer, number of nucleated cells per ml was obtained by the equation:

$\frac{cell\ count}{8} \times dilution\ factor \times 10^4$. The fold expansion of total nucleated cells was calculated by dividing the number of nucleated cells after culture by the number of nucleated cells at day 0. The percentages of $CD34^+$, $CD34^+CD38^-$ and $CD61^+CD41^+$ cells were determined by flow cytometry as described in Section 3.6.

Assay of CFU-MK was performed using the methylcellulose system (Li *et al.* 2000). Enriched $CD34^+$ cells or expanded cells at 3×10^3/ml were cultured in triplicate in

1% methylcellulose (Sigma) in IMDM supplemented with 20 ng/ml TPO, 30% FCS, 1% BSA and 0.1 mM β-mercaptoethanol (β-ME) (Gibco). The cultures were incubated for 12 days. The colonies were examined under an inverted microscope. A CFU-MK colony was defined as a cluster of three or more megakaryocytes (Yang *et al.* 1995).

3.4.1.2 Flt-3 Ligand Receptor Assay

3.4.1.2.1 Culture of Human Megakaryoblastic Cell Lines

Four human megakaryoblastic cell lines, Meg-01, CHRF-288-11, Dami and M-07e, were cultured in IMDM containing 10% FCS. IL-3 at 25 ng/ml (Genzyme; Cambridge, MA, USA) was added to M-07e. All cultures were maintained at 37°C in a 5% CO_2 humidified incubator. When the cell densities of cultures reached 1×10^6/ml, cells were washed with IMDM without FCS and centrifuged at 200 × g for 10 min at 4°C. Cells were then cultured in IMDM with 10% FCS at 5×10^4/ml.

3.4.1.2.2 Identification of Flt-3 Ligand Receptors

Four cultured cell lines and MNC from three pediatric acute lymphoblastic leukemia patients at 5×10^5 per 100 µl were stained with phycoerythrin (PE)-conjugated mouse anti-human FL receptor monoclonal antibody (Immunotech; Marseille, France). MNC from acute lymphoblastic leukemia patients were obtained by density gradient centrifugation over Ficoll Hypaque. Another 5×10^5 cells of each sample were stained with isotypic IgG control (Immunotech). All samples were incubated for 20 min in the dark at room temperature. They were washed with 2 ml PBS and centrifuged at 200 × g at 20°C for 6 min. Twenty thousand events were acquired for each sample. These cells were analyzed using a FACScan flow cytometer and the

Lysis II software (BD). Cell debris was excluded by gating on the forward and side scatter profile during data analysis.

3.4.2 Effects of Mannose-Binding Lectin on the *Ex Vivo* Expansion of Hematopoietic Stem and Progenitor Cells

3.4.2.1 Ex Vivo Expansion of Cord Blood $CD34^+$ Cells with Mannose-Binding Lectin

Enriched $CD34^+$ cells from eight CB samples at 2×10^4/ml were cultured in a 24-well culture plate in 1 ml IMDM supplemented with 10% FCS. Two groups of cytokine treatments were used: (A) for the megakaryocytic lineage: 50 ng/ml TPO, 25 ng/ml SCF, 20 ng/ml IL-1β, 25 ng/ml IL-6 and 25 ng/ml IL-11; (B) for the early progenitor cells: 50 ng/ml TPO, 25 ng/ml SCF and 50 ng/ml FL. MBL at 0, 50, 100, 200, 400 and 800 ng/ml were added to the cultures in both groups (A) and (B). The cultures were incubated at 37°C and 5% CO_2 in a fully humidified atmosphere for 35 days. At days 7, 14, 21 and 28, half of cells from each culture was harvested and topped up with the same volume of fresh medium and cytokines. Cell counts, viability, flow cytometry analysis ($CD34^+$, $CD34^+CD38^-$ and $CD61^+CD41^+$ cells) and colony-forming assays as described in Section 3.5 were performed at days 14, 21, 28 and 35.

3.4.2.2 Effects of Mannose-Binding Lectin on the Preservation of Early Stem and Progenitor Cells

MNC at 2×10^5/ml or enriched $CD34^+$ cells at 2×10^4/ml were cultured in a 25 cm^2 flask (Corning; New York, NY, USA) in 5 ml X-Vivo 10. In each culture, 100, 200, 400, 800, 1600 ng/ml MBL, 40 ng/ml FL, 40 ng/ml FL plus 200 ng/ml MBL were added. The cells cultured in the absence of MBL and FL were used as the control.

The cultures were incubated for 35 days without any medium changes. At days 14, 21, 28 and 35, 1 ml of cells were harvested for cell counts, cell viability test and colony-forming assays. Eight independent CB cultures using MNC or $CD34^+$ cells were performed.

3.4.2.3 Transplantation of Expanded Cells into NOD/SCID Mice

Enriched $CD34^+$ cells at 2×10^4/ml from nine fresh CB samples were cultured in 5 ml IMDM + 10% FCS in the presence of 50 ng/ml TPO, 25 ng/ml SCF and 50 ng/ml FL with or without 200 ng/ml MBL for 14 days. At day 7, another 5 ml fresh medium with cytokines were added to the cultures. Cells were harvested at day 14 and transplanted into NOD/SCID mice. The transplantation of expanded human cells into NOD/SCID mice was described in details in Section 3.7.

3.4.3 Optimization of Culture Duration, Culture Media, Autologous Plasma and Cytokine Combinations for the Preclinical *Ex Vivo* Expansion of Hematopoietic Stem and Progenitor Cells

3.4.3.1 Comparison of Culture Duration, Culture Media and Cytokine Combinations

Ten independent CB cultures were performed for the comparison of culture duration, various media and cytokine combinations. Enriched $CD34^+$ cells at 2×10^4/ml were cultured in a 24-well culture plate. The four media being assayed were IMDM supplemented with 10% FCS, X-Vivo 10 (Biowhittaker; Walkersville, MD, USA), QBSF-60 and StemSpan SFEM (Stem Cell Technologies; Vancouver, BC, Canada). The latter three media were serum-free and were supplemented with 1% HSA, 2 mM L-glutamine (Gibco) and 0.1 mM β-ME. As recommended by the manufacturer, 40 μg/ml low density lipoprotein (Sigma) was added into StemSpan medium. For this

preclinical study, cytokines of clinical or preclinical grades were assessed. Five cytokines were used: 50 ng/ml TPO, 50 ng/ml SCF, 80 ng/ml FL, 40 ng/ml G-CSF and 100 ng/ml IL-6. The four cytokine combinations were TPO, SCF and FL (TSF), plus either G-CSF (TSFG), IL-6 (TSF6) or both cytokines (TSFG6). All cytokines were purchased from Peprotech, except for G-CSF which was purchased from Roche (Neupogen; Basle, Switzerland). The cultures were incubated at 37°C and 5% CO_2 in a fully humidified atmosphere for 12 days. At days 4 and 8, each culture was split into three portions and topped up with the same volume of fresh media and cytokines. Cell counts, viability, flow cytometry analysis ($CD34^+$, $CD34^+CD38^-$, $CD61^+CD41^+$ cells) and colony-forming assays were performed at days 8 and 12.

3.4.3.2 Effects of Autologous Cord Blood Plasma

Another six CB cultures were performed to investigate the effects of autologous CB plasma on the expansion of $CD34^+$ cells. In addition to the same media and cytokine conditions described in Section 3.4.3.1, 10% autologous CB plasma was included in the three serum-free media. In the IMDM cultures, 10% FCS was replaced by 10% autologous CB plasma. To obtain autologous CB plasma, 10 ml of each CB sample was centrifuged at 3,000 × g for 15 min at room temperature. After the collection of supernatant, the plasma was stored at –20°C and heat inactivated at 56°C for 30 min before use.

3.4.3.3 Effects of Flt-3 Ligand and Dosage of Thrombopoietin and Stem Cell Factor

Eight independent CB cultures were performed to elucidate the effects of FL and different doses of TPO and SCF on the expansion outcomes. Enriched $CD34^+$ cells at 2×10^4/ml were cultured in QBSF-60 in the presence of TPO and SCF at 50 ng/ml or

100 ng/ml, 40 ng/ml G-CSF, with or without 80 ng/ml FL for 12 days. At days 4 and 8, cultures were split into three portions and topped up with two-third volume of fresh medium and cytokines. At days 8 and 12, expanded cells were harvested for analysis. The same parameters as those in Section 3.4.3.1 were analyzed.

3.4.3.4 Transplantation of Expanded Cells into NOD/SCID Mice

Enriched $CD34^+$ cells from five cryopreserved CB at 2×10^4/ml were cultured in QBSF-60 with 50 ng/ml PEG-MGDF (Kirin), 50 ng/ml rhSCF (Kirin), 40 ng/ml G-CSF, with or without 80 ng/ml FL for 12 days. Expanded cells were then transplanted into NOD/SCID mice studying the effects of FL on the engrafting ability of hematopoietic stem and progenitor cells.

Section 3.5 Progenitor Colony-Forming Assays

3.5.1 Colony-Forming Unit Assay

Colony-forming unit granulocyte-macrophage (CFU-GM), burst-forming unit/colony-forming unit erythroid (BFU/CFU-E) and colony-forming unit granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM) were assayed in 1% methylcellulose culture in IMDM supplemented with 30% FCS, 1% BSA and 0.1 mM β-ME in the presence of 3 UI/ml EPO (Eprex, Cilag; Zug, Switzerland), 10 ng/ml GM-CSF (Leucomax; Basle, Switzerland), 10 ng/ml IL-3 (Peprotech) and 50 ng/ml SCF (Peprotech). Enriched or expanded $CD34^+$ cells at 3×10^3/ml, or fresh or expanded MNC at 5×10^4/ml were seeded in triplicate and incubated for 14 days. Colonies were scored using an inverted light microscope by their morphology. Colonies of more than 50 cells were counted as a CFU and the following categories of CFU were classified (Eaves *et al.* 1995).

(i) CFU-GM

Clonogenic progenitors of granulocytes and macrophages (CFU-GM) were defined on the basis of their ability to produce colonies containing 50 or more of these cells. Morphologically, CFU-GM showed a relatively flat, non-hemoglobinized and homogeneous morphology, often with a more concentrate central core of granulocytes surrounded by a less dense halo of macrophages.

(ii) BFU/CFU-E

BFU-E was distinguished by its greater proliferative capacity of giving rise to larger, densely packed, multi-clustsered erythroid colonies when compared to those produced from CFU-E. It was orange to dark red hemoglobinized cells with no appearance of translucent cells. A BFU-E colony was identified as 5 or more clusters of cells.

CFU-E-derived colonies were identified as one or two clusters containing up to a maximum of approximately 100 to 200 erythroblasts. Each cluster contained a minimum of eight erythroblasts. The erythroblasts were recognized as a distinctive reddish-orange hue due to their content of hemoglobin.

(iii) CFU-GEMM

CFU-GEMM was identified as colonies containing cells of multiple lineages appeared as small numbers of granulocytes, macrophages and/or megakaryocytes around the periphery of a spherical mass of hemoglobinized erythroid cells. CFU-GEMM were examined under high power, to avoid being mistakenly scored as pure erythroid colonies.

3.5.2 Colony-Forming Unit-Megakaryocyte

CFU-MK, unless specified, was assayed using a plasma clot system. Enriched or expanded $CD34^+$ cells at 3×10^3/ml, or fresh or expanded MNC at 5×10^4/ml were grown in duplicate in IMDM containing 10% citrated bovine plasma (Sigma), 10% FCS, 1% BSA, 0.1 mM β-ME, 0.34 mg/ml calcium chloride in the presence of 50 ng/ml TPO and 20 ng/ml IL-3. After 12 days of incubation, the clots were air-dried and fixed with 1% paraformaldehyde (Sigma) pH 7.4 in PBS. They were stored at 4°C before scoring. Immunofluorescence staining was performed by labeling with monoclonal antibody CD61-fluorescein isothiocyanate (FITC) (Dako; Boston, MA, USA) (Kratz-Albers *et al.* 2000). The clots were rinsed with 3 ml PBS with 0.5% BSA. CD61-FITC monoclonal antibody was diluted 1:10 in PBS and 500 µl diluted antibody was then added onto the clots and incubated in the dark at room temperature for 20 min. The clots were rinsed with 3 ml PBS with 0.5% BSA. CFU-MK was identified as a cluster of three or more strongly-stained CD61 positive cells examined under a fluorescent microscope.

3.5.3 Calculations of CFU

The number of CFU generated from the culture was calculated by the equation:

$$\frac{total\ nucleated\ cells}{no.of\ cells\ seeded} \times no.of\ colonies\ formed.$$

The fold expansion of CFU was obtained by dividing the number of CFU after expansion by the number of CFU at day 0.

Section 3.6 Flow Cytometry Analysis

At day 0, 1×10^5 enriched $CD34^+$ cells were stained with 10 µl each of CD34-FITC (Clone 8G12) (BD; San Jose, CA, USA), CD38-phycoerythrin (PE) (BD) and respective isotypic control monoclonal antibodies (BD). CD34-FITC monoclonal antibody (Anti-HPCA-2) recognized different epitopes of the CD34 antigen from that recognized by the monoclonal antibody QBEND/10 used in Section 3.3 for $CD34^+$ cell enrichment. The cells were incubated in the dark for 20 min at room temperature. After incubation, 2 ml PBS with 0.5% BSA was added. The cells were centrifuged at 200 × g at room temperature for 6 min and resuspended in 0.5 ml 1% paraformaldehyde (Sigma) in PBS pH 7.4. Fixed cells were analyzed within 30 min or stored at 4°C for less than 4 hr before acquisition. Thirty thousand events were acquired using a FACSCalibur flow cytometer (BD) for the determination of the purity of enriched $CD34^+$ cells. These results were analyzed using the CellQuest software (BD) with dead cells and debris gated out by the forward and side scatter profiles.

After expansion, cultured cells were washed with PBS containing 0.5% BSA and centrifuged at 300 × g at room temperature for 10 min. Cells were resuspended in 100 µl PBS and stained with 10 µl each of CD34-FITC/CD38-PE (BD), CD61-FITC/CD41-PE (Dako), and their respective isotypic controls for 20 min in the dark at room temperature. Cells were then washed and resuspended in 100 µl PBS. Prior to analysis, 10 µl 7-amino-actinomycin D (7-AAD) (Pharmingen) was added to each sample and incubated for 10 min in the dark at room temperature. Samples were washed with 2 ml PBS and centrifuged at 200 × g for 6 min at room temperature. The cells were resuspended in 500 µl 1% paraformaldehyde. Sixty thousand events

were acquired for each sample. These cells were analyzed using a FACSCalibur flow cytometer and the CellQuest software (BD). Dead cells which were 7-AAD positive were gated out during data analysis.

The absolute numbers of $CD34^+$, $CD34^+CD38^-$ and $CD61^+CD41^+$ cells were calculated by multiplying their respective percentages obtained from flow cytometry analysis by number of total nucleated cells. Their fold expansion was calculated by dividing their numbers after culture by the numbers at day 0.

Section 3.7 Transplantation of Non-Obese Diabetic/Severe Combined Immunodeficient Mice

This study was approved by the Animal Research Ethics Committee of The Chinese University of Hong Kong. Non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice, which were characterized by the lack of mature lymphocytes, macrophage dysfunction, and an absence of circulating complements (Koyanagi *et al.* 1997), were obtained from The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia. The mice were bred in microisolator cages in the Laboratory Animal Services Centre at The Chinese University of Hong Kong. These animals were housed in laminar flow racks under pathogen-free conditions and fed with autoclaved food and water. At 6 – 8 weeks of age, the mice were exposed to 300 cGy of gamma irradiation using the Gammacell-1000 Elite Irradiator (MDS Nordion, Kanata, On, Canada). They were weighed and anesthetized with a mixture of ketamine at 50 mg per kg of body weight and xylazine HCl at 25 mg/kg by intraperitoneal injection. The cells for transplantation were washed with IMDM and centrifuged at 300 × g at room temperature for 10 min. These cells at 1×10^5/ml were

resuspended in 1ml IMDM. Then, 2 – 4 × 10^4 enriched $CD34^+$ cells or expanded cells which were derived from 2 – 4 × 10^4 cells at day 0 were injected into the lateral tail vein of the mice.

After 6 weeks, the mice were sacrificed by cervical dislocation. BM (equivalent of 2 femurs), PB and spleen were collected for analysis. Single-cell suspension of BM was prepared by aspirating the femurs with PBS containing 5% FCS to release BM cells followed by sedimentation to get rid of clumps and debris. Cells were then processed for flow cytometry analysis, PCR and CFU assays. The spleen of each transplanted mice was homogenized in PBS containing 5% FCS before cell harvesting. PB was collected by heart puncture. Red blood cells were lyzed with 0.9% ammonium chloride for 5 min. Cells were then washed and resuspended in PBS containing 5% FCS for flow cytometry analysis.

Section 3.8 Assessment of Human Cell Engraftment in Transplanted NOD/SCID Mice

3.8.1 Flow Cytometry Analysis

Cell samples at 5 × 10^5/200 μl in PBS containing 5% FCS were incubated with 10 μl mouse anti-human CD45-phycoerythrin-cyanine 5-succinimidylester (PC5) antibody (Immunotech) and propidium iodide (PI) at 10 μg/ml (Sigma) for 20 min in the dark at room temperature. The control tube contained the isotypic antibodies (Immunotech) and PI. After washing with PBS, cells were analyzed on a FACScan Flow Cytometer (BD) using the Lysis II program with the non-viable cells (PI positive) being gated out. Specific subsets of human hematopoietic cells were determined when BM cells contained more than 1% of human $CD45^+$ cells. Cell

samples were stained with 10 μl CD45-PC5, PI and CD34-FITC, CD33-FITC, CD14-FITC, CD19-FITC or CD61-FITC. CD33-FITC, CD14-FITC and CD19-FITC monoclonal antibodies were purchased from Pharmingen. All antibodies were tested to be non-cross-reactive with the NOD/SCID mouse surface antigens. Seventy-eight thousand events were acquired for each sample.

3.8.2 PCR Analysis

To confirm the engraftment of human cells in a murine background, genomic DNA from mouse BM cells was extracted by a DNeasy DNA purification kit (Qiagen, Hilden, Germany). BM cells were collected and washed with PBS. The supernatant was discarded. Cell pellets were stored at -80°C before extraction. Cells were thawed at room temperature until the pellet can be dislodged by gently flicking the tube. According to the manufacturer's instruction, 5×10^6 BM cells were centrifuged at 300 × g for 5 min at room temperature and resuspended in 200 μl PBS. Twenty μl proteinase K and 200 μl Lysis Buffer AL were added to the sample, mixed thoroughly by vortexing and incubated at 70°C for 10 min. The buffer was optimized to allow direct cell lysis. After incubation, 200 μl absolute ethanol was added to the lysate. Wide pore pipette tips were used from this step onwards to avoid the genomic DNA from breaking down. The lysate was then loaded onto a spin column sitting in a 2 ml collection tube and centrifuged at 6,000 × g for 1 min at room temperature. Extracted DNA was able to bind to the silica-gel membrane inside the column. The collection tube and the flow through materials were discarded. The column was placed in another 2 ml collection tube and 500 μl Buffer AW1 was added. It was centrifuged at 6,000 × g for 1 min at room temperature. The collection tube and the flow through were again discarded. The column was put in another 2 ml collection

tube. Buffer AW2 (500 µl) was added this time. The column was centrifuged at 20,000 × g for 3 min at room temperature. It was removed and placed in a 1.5 ml microcentrifuge tube. Buffer AE (200 µl) was pipetted onto the membrane of the column followed by a 1 min incubation at room temperature. Buffer AE was used to dissolve DNA bound to the membrane. The column was centrifuged at 6,000 × g for 1 min to elute dissolved DNA. Another 200 µl Buffer AE was added to the membrane. The incubation and centrifugation steps were repeated to elute residual DNA.

The presence of human-specific DNA in BM of transplanted mice was confirmed by polymerase-chain reaction (PCR) amplification using primers complementary to the sequence of an 850-bp DNA fragment of the human-specific alpha satellite DNA of chromosome 17 (Waye *et al.* 1986). Amplification of the centromere-specific human fragments of chromosome 17 was performed using primers corresponding to the primer pair 17a1/17a2 as described by Warburton *et al.* (1991). The 5' primer (5' GGG ATA ATT TCA GCT GAC TAA ACA G 3') covered the positions 15 to 39, and the 3' primer (5' TTC CGT TTA GTT AGG TGC AGT TAT C 3') covered the positions 867 to 891 of the sequence HSSATA17 (Gene Bank #M13882). For PCR, Taq polymerase and reagents were purchased from Gibco. The PCR reaction contained 1X PCR buffer, 200 µM dNTP, 250 nM of each primer, 2 mM $MgCl_2$ and 250 ng genomic DNA (Möbest *et al.* 1999). Samples were initially denatured at 94°C for 10 min. Amplification began with denaturation at 94°C for 1 min, 66°C annealing for 1 min, 72°C extension for 1 min for 35 cycles, followed by final elongation at 72°C for 10 min. Genomic DNA samples from human CB and BM cells of non-transplanted NOD/SCID mouse were processed in parallel as positive and negative

controls, respectively. For the positive control, 0.1, 1.0, 5.0, 10.0, 30.0, 50.0% of human CB DNA mixed with mouse DNA were included as a set of standards. Amplified PCR products were electrophoresed through 1.75% agarose gels in 1X TAE buffer (0.04M Tris-acetate, 0.001M EDTA pH8) with the power supply of 100 Volts for 45 min. The agarose gel was pre-stained with ethidium bromide before electrophoresis and visualized under ultraviolet light.

Section 3.9 Statistical Analysis

The SigmaStat software (Jandel Scientific Software, San Rafael, CA) was used for statistical analysis. For the comparison of the effects of FL and SCF on the expansion of megakaryocytic progenitor cells and the optimization of culture conditions, paired t-test was used in case of normal distribution or Wilcoxon Sign Rank test was used in case of non-parametric distribution. One-way ANOVA and paired t-test or Wilcoxon Sign Rank test, whichever suitable, were performed for investigating the effects of MBL on the *ex vivo* expansion of hematpoietic stem and progenitor cells. A p-value equal to or less than 0.05 was considered as statistical significant. Data were expressed as mean ± standard error of mean (S.E.).

CHAPTER FOUR

EFFECTS OF FLT-3 LIGAND AND STEM CELL FACTOR ON THE EXPANSION OF MEGAKARYOCYTIC PROGENITOR CELLS

Section 4.1 Results

4.1.1 *Ex Vivo* Expansion of $CD34^+$ Cells

At day 0, the purity of enriched $CD34^+$ cells were 87.3 ± 4.60% (range 88.7 – 99.1%) (Figure 4.1) and the viability of $CD34^+$ cells as determined by the trypan blue dye exclusion assay was 99.0 ± 0.44% (range 97.0 – 100%). At days 7, 14, and 21, cell viability were 95.7 ± 0.49% (range 94.0 – 98.0%), 95.8 ± 0.72 (range 93 – 98.5%) and 94.5 ± 0.79% (range 91.8 – 97.7%), respectively. Enriched $CD34^+$ cells were significantly expanded in the presence of FL or SCF, in combination with TPO, IL-3 and IL-6. There were overall increases of nucleated cells, $CD34^+$ cells, $CD34^+CD38^-$ cells, $CD61^+41^+$ cells as well as CFU-MK. Replacing FL with SCF significantly reduced the levels of all cell parameters.

Total Nucleated Cells

The expansion of total nucleated cells was in general more efficient in cultures containing FL when compared to those containing SCF (Table 4.1). The fold increases of total nucleated cells in cultures with FL were 114%, 128% and 231% of those with SCF at days 7, 14, and 21, respectively. The number of total nucleated cells expanded in FL-containing culture was significantly higher than that in SCF-containing culture at day 21 ($p = 0.0417$) (Figure 4.2).

$CD34^+$ Cells and $CD34^+CD38^-$ Cells

The percentage of $CD34^+$ cells decreased with the duration of culture. An increase in absolute numbers of $CD34^+$ cells was seen at day 14 (Table 4.1). Significantly higher percentage ($p = 0.005$) (Figure 4.3) and fold increase ($p = 0.0273$) (Figure 4.4) of $CD34^+$ cells were observed in cultures with FL at day 14 when compared to those with SCF. At day 21, the fold increase of $CD34^+$ cells in cultures with FL was also significantly higher than that with SCF ($p = 0.0159$) (Figure 4.4). The yields of $CD34^+$ cells in cultures with FL were 123%, 212% and 299% of those with SCF.

FL supported more efficient expansion of early progenitors than SCF. A significant difference was observed in the fold increase of $CD34^+CD38^-$ cells at day 7 ($p = 0.0195$) (Figure 4.5) and the same trend was observed at day 14 ($p = 0.0658$) and at day 21 ($p = 0.0683$) (Table 4.1 and Figure 4.3). The fold increases of $CD34^+CD38^-$ cells in cultures with FL were 173 – 468% of those with SCF.

Megakaryocytic Progenitors

For the megakaryocytic lineage, significant differences were observed in the yield of $CD61^+CD41^+$ cells ($p = 0.0283$) (Table 4.1) and the fold increase of CFU-MK at day 14 ($p = 0.0166$) (Figure 4.6). FL appeared to support better expansion of megakaryocytic progenitors than SCF. $CD61^+CD41^+$ cells (Figure 4.7) and CFU-MK in FL-containing cultures were 136 – 182% and 226 – 200% of those in SCF-containing cultures, respectively (Table 4.1).

Duration of Culture

The culture for 14 days appeared to be the most effective duration for the expansion of both primitive and megakaryocytic progenitors (Table 4.1). The absolute numbers of $CD34^+$ cells, $CD34^+CD38^-$ cells, $CD61^+CD41^+$ cells and CFU-MK peaked at day 14. Instead of being depleted, $CD34^+$ cells increased to 23.8- and 10.7-fold in cultures with FL and SCF respectively whereas $CD34^+CD38^-$ cells increased to 33.9- and 10.7-fold after 14 days of culture. $CD61^+CD41^+$ cells cultured with FL and SCF were 15.6% and 9.78% of the total cell populations at day 14 (Figure 4.7). The efficiency of the culture system was again demonstrated by the 584-fold increase of CFU-MK in cultures with FL at day 14 (Figure 4.6).

4.1.2 Identification of Flt-3 Ligand Receptors

The expressions of FL receptor on four megakaryoblastic cell lines and bone marrow cells from three pediatric acute lymphoblastic leukemia patients were investigated by flow cytometry. As shown in Figure 4.8, all four cell lines did not show any positive expression of FL receptors while one leukemic sample expressed a high level of receptors.

Section 4.2 Discussion

The effectiveness and possible mechanism of using FL and SCF, in combination with TPO, IL-3 and IL-6 on the *ex vivo* expansion of cord blood $CD34^+$ cells to the megakaryocytic lineage were studied. Results indicated that FL was significantly more effective than SCF in the overall expansion of hematopoietic cells including early progenitors and megakaryocytes.

Bertolini *et al.* (1997a) reported the clinical transplant of expanded PBSC and demonstrated that platelet transfusion support was not required in patients who received the highest doses of cultured $CD61^+$ cells. In our system, FL plus TPO, IL-3 and IL-6 could support up to 584-fold increase of CFU-MK at day 14 of culture. $CD61^+CD41^+$ cells were over 15% of total nucleated cells in the culture, representing a substantial proportion of megakaryocytic progenitors. The data compared favorably with those reported by other groups using different cytokine combinations and cell sources (Piacibello *et al.* 1996b, Bertolini *et al.* 1997a, Williams *et al.* 1998, Lefebvre *et al.* 2000, van den Oudenrijn *et al.* 2000). They reported the expansion of CFU-MK from 17- to 200-fold.

Previous studies showed that the expansion outcomes of megakaryocytic progenitors were influenced by the source of $CD34^+$ cells (van den Oudenrijn *et al.* 2000) and cytokine combinations (van den Oudenrijn *et al.* 1999, Drayer *et al.* 2000, Lefebvre *et al.* 2000, Su *et al.* 2001). Reports on effects of FL and SCF on the expansion of hematopoietic cells to the megakaryocytic lineage have not been consistent (Shapiro *et al.* 1996, Ohmizono *et al.* 1997, Williams *et al.* 1998). Ohmizono *et al.* (1997) demonstrated that FL-containing cultures with TPO, IL-3 IL-6 and IL-11 produced more CFU-MK when compared with SCF-containing cultures. However, Williams *et al.* (1998) observed better expansion efficiencies using SCF in cultures with TPO, IL-1, IL-6 and IL-11. In our culture system using enriched CB $CD34^+$ cells, FL was superior to SCF in the expansion of $CD61^+CD41^+$ cells and CFU-MK.

The yields of both early and megakaryocytic progenitors peaked at day 14. The prolonged expansion to 21 days increased total nucleated cells but reduced $CD34^+$

cells, $CD61^+CD41^+$ cells and CFU-MK. It was significant that our culture conditions promoted the expansion of megakaryocytic progenitors and simultaneously expanded $CD34^+$ cells and $CD34^+CD38^-$ cells to 23.8- and 33.9-fold, respectively. In our culture conditions, FL significantly improved the expansion of $CD34^+$ cells when compared to SCF (Figure 4.4). Our findings are in agreement with those of Kobari *et al.* (1998) who demonstrated that the expansion of BM $CD34^+$ cells was increased by 4.4-fold when FL was added to the culture system with SCF, IL-3 and IL-6. Capmany *et al.* (1999) also reported that the addition of FL to the same cytokine combination increased the expansion of CB $CD34^+$ cells by 2.7-fold. Piacibello *et al.* (1997) described a culture system with two cytokines, FL and TPO, which was capable of sustaining proliferation and renewal of primitive progenitors for more than six months.

The mechanism of FL on the megakaryocytic lineage remains unclear. Ratajczak *et al.* (1996) and Turner *et al.* (1996) showed that FL alone or in combination with cytokines such as TPO had no effect on *in vitro* megakaryocytopoiesis and megakaryocyte ploidy. In addition, the flt-3 knockout mice did not show any defects in megakaryocyte and platelet production (Mackarehtschian *et al.* 1995). Considering the lack of FL receptors on four megakaryocytic cell lines, the effect of FL on promoting expansion of megakaryocytic progenitors might possibly be mediated by its early action at the multipotent stem cell stage. It has been suggested that early hematopoietic cells ($CD34^+CD38^-$ cells) were more responsive to FL than committed progenitor cells ($CD34^+CD38^+$ cells) (Dooley *et al.* 1997).

In summary, our data demonstrated that in comparison with SCF, FL was a more effective cytokine for augmenting TPO, IL-3 and IL-6 on the expansion of CB $CD34^+$ cells to megakaryocytic progenitors. The conditions did not deplete primitive progenitors, thus representing a potentially useful system for the clinical application of expanding CB hematopoietic stem and progenitor cells for BMT.

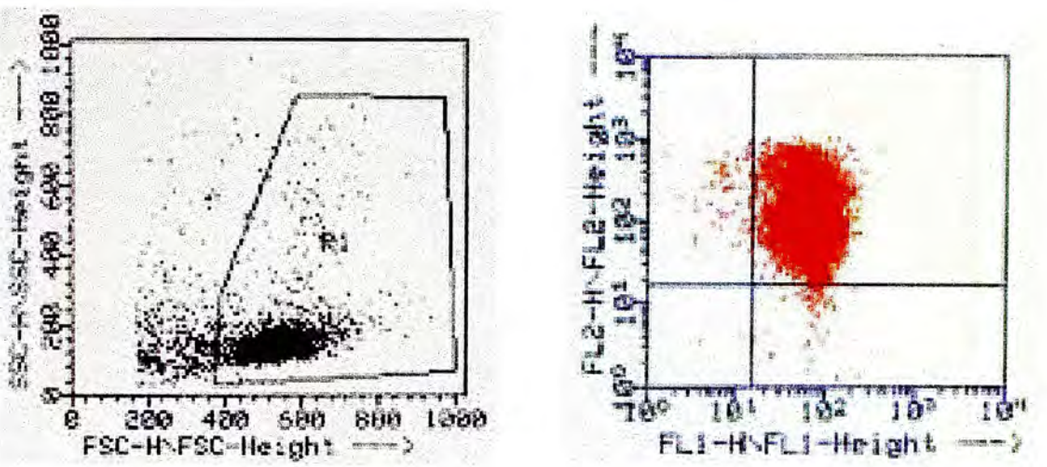


Figure 4.1 Flow Cytometry Analysis: Determination of the Purity of $CD34^+$ Cells after Enrichment

Enriched $CD34^+$ cells were stained with CD34-FITC (FL1) and CD38-PE (FL2) monoclonal antibodies. Cells in the upper and lower right quadrants were CD34-positive and in the lower right quadrants were $CD34^+CD38^-$.

Table 4.1 *Ex Vivo* Expansion of Cord Blood $CD34^+$ Cells: Effects of Flt-3 Ligand and Stem Cell Factor

	Day 7		Day 14		Day 21	
	FL	SCF	FL	SCF	FL	SCF
Total Nucleated Cells ($\times 10^6$)	1.83 ± 0.66	1.72 ± 0.64	14.6 ± 4.96	12.5 ± 4.28	16.0 ± 4.62*	10.9 ± 3.71*
$CD34^+$ Cells (%)	13.6 ± 2.17	12.4 ± 2.37	6.51 ± 2.35**	3.74 ± 1.93**	1.61 ± 0.61	1.11 ± 0.49
$CD34^+$ Cells ($\times 10^4$)	26.0 ± 1.16	21.2 ± 8.74	112 ± 68.0	52.9 ± 34.9	24.5 ± 12.8	8.20 ± 4.39
$CD34^+CD38^-$ Cells (%)	1.33 ± 0.63	0.84 ± 0.38	0.54 ± 0.47	0.24 ± 0.18	0.07 ± 0.07	0.02 ± 0.01
$CD34^+CD38^-$ Cells ($\times 10^4$)	2.84 ± 1.62	1.60 ± 0.88	10.8 ± 1.16	3.94 ± 0.32	1.32 ± 0.12	0.88 ± 0.14
$CD61^+CD41^+$ Cells (%)	18.5 ± 5.61	15.7 ± 4.50	15.6 ± 5.05	9.78 ± 2.93	2.51 ± 0.80	2.24 ± 0.84
$CD61^+CD41^+$ Cells ($\times 10^5$)	4.44 ± 1.91	3.27 ± 1.39	27.7 ± 11.1*	15.2 ± 6.08*	3.29 ± 0.93	2.21 ± 1.06
CFU-MK ($\times 10^4$)	2.59 ± 1.10	2.25 ± 0.99	18.3 ± 7.00	12.1 ± 3.83	9.95 ± 4.85	4.98 ± 1.97

Enriched $CD34^+$ cells at 8×10^4/ml were expanded in the presence of TPO, IL-3, IL-6 plus either FL or SCF for 21 days. Results were expressed as mean ± S.E. of cell populations expanded from 1 ml of $CD34^+$ cells from Day 0.

n = 7, * $p < 0.05$, ** $p < 0.001$

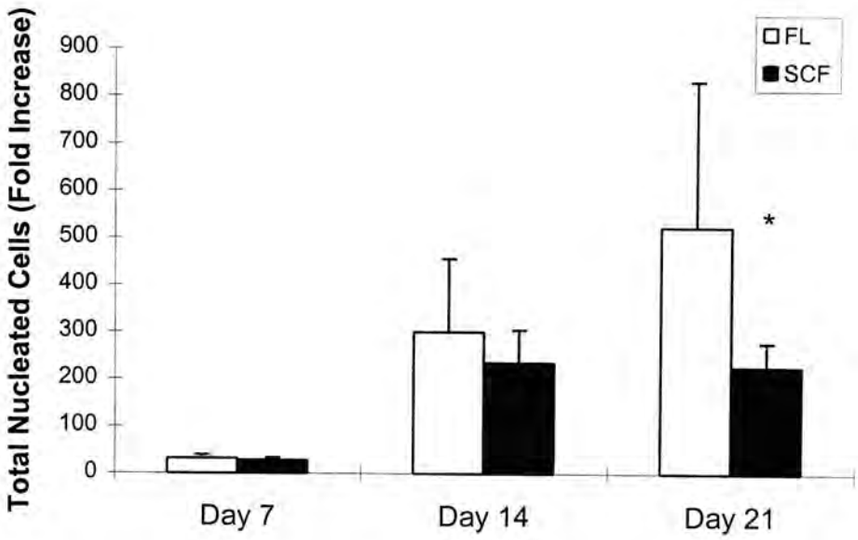


Figure 4.2 **Effects of FL and SCF on the Fold Increase of Total Nucleated Cells**

Enriched $CD34^+$ cells at 8×10^4/ml were cultured in 1 ml IMDM + 10% FCS in the presence of TPO, IL-3, IL-6 plus either FL or SCF, each at 20 ng/ml for 21 days. FL supported more efficient expansion of total nucleated cells when compared with SCF. Results were presented as mean ± S.E. n = 7, * $p < 0.05$.

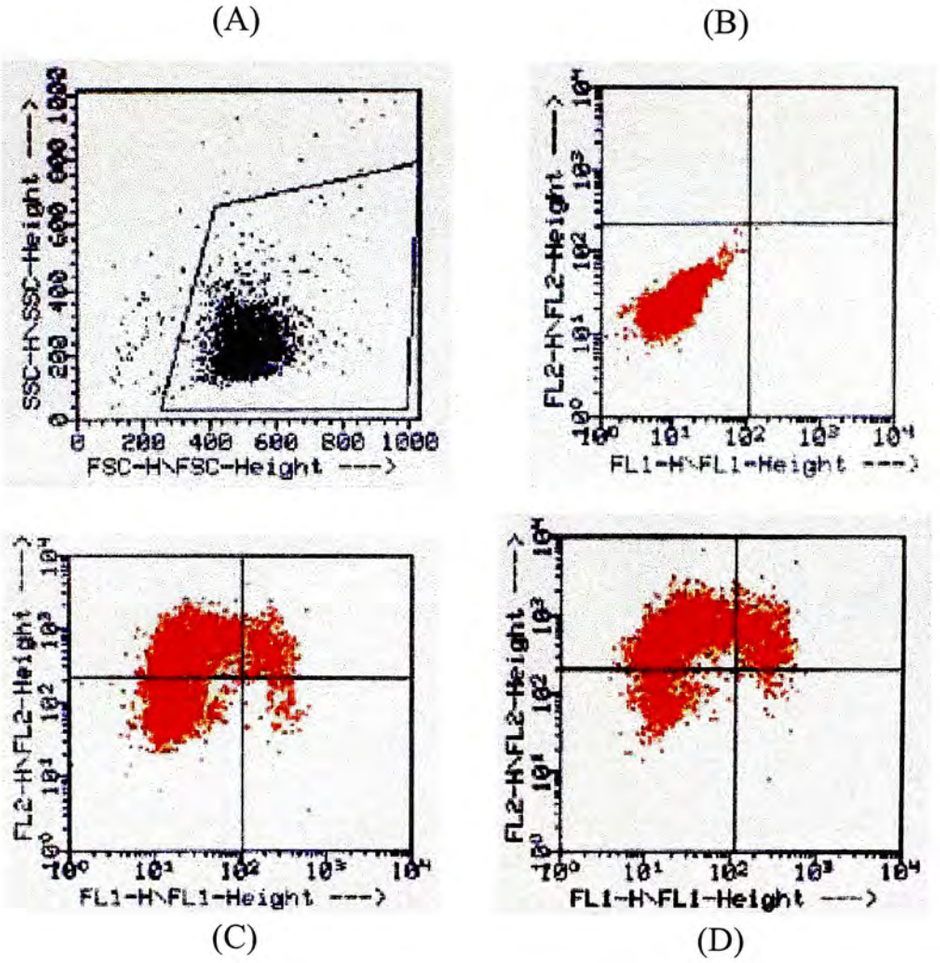


Figure 4.3 **Flow Cytometry Analysis of Expanded Cells: Determination of the Percentages of $CD34^+$ Cells and Subsets**

Cells were expanded in cultures with FL (C) or SCF (D). Cell debris were gated out using their FCS/SSC profile (A). $CD34^+$ cells and $CD34^+CD38^-$ cells were determined by their binding to CD34-FITC and CD38-PE antibodies (C & D) in comparison with the respective isotypic controls (B).

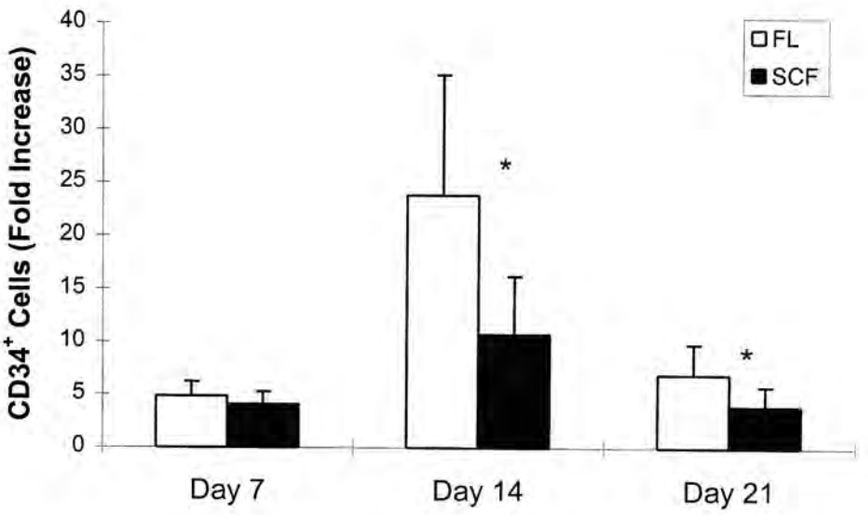


Figure 4.4 **Effects of FL and SCF on the Fold Increase of $CD34^+$ Cells**

$CD34^+$ cells were cultured in either FL or SCF with TPO, IL-3 and IL-6 for 21 days. $CD34^+$ cells were analyzed by flow cytometry. Fold increases of $CD34^+$ cells in FL cultures were significantly higher than those in SCF cultures at days 14 and 21. Results were presented as mean ± S.E. $n = 7$, * $p < 0.05$.

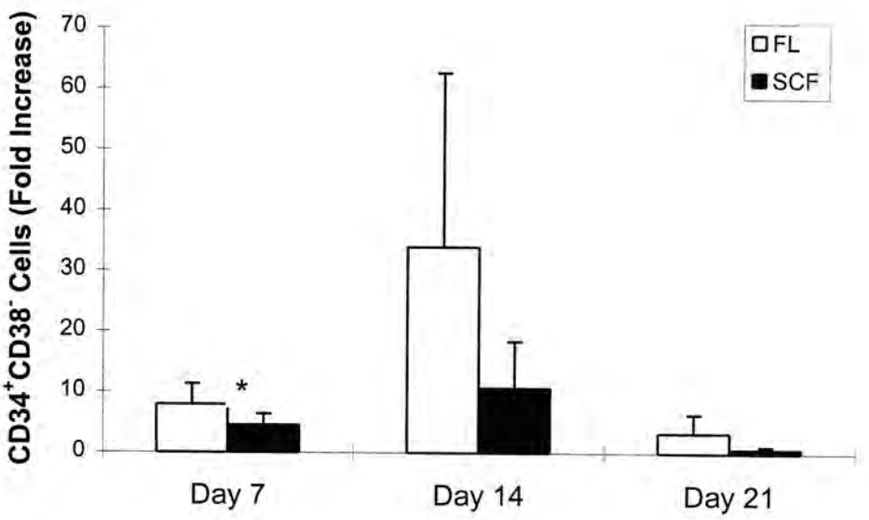


Figure 4.5 **Effects of FL and SCF on the Fold Increase of $CD34^+CD38^-$ Cells**

$CD34^+$ cells were cultured in either FL or SCF with TPO, IL-3 and IL-6 for 21 days. $CD34^+CD38^-$ cells were analyzed by flow cytometry. The fold increase of $CD34^+CD38^-$ cells in the presence of FL was significantly higher than that in the presence of SCF at day 7. Results were presented as mean ± S.E. n = 7, * $p < 0.05$.

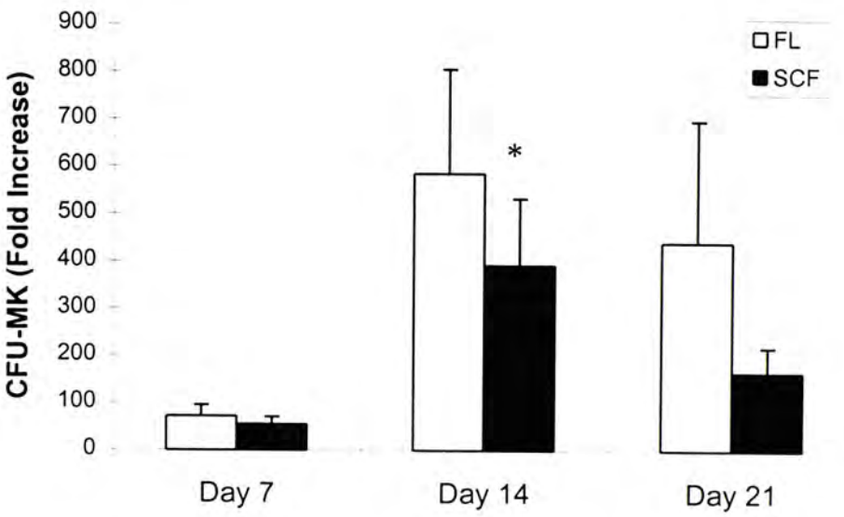
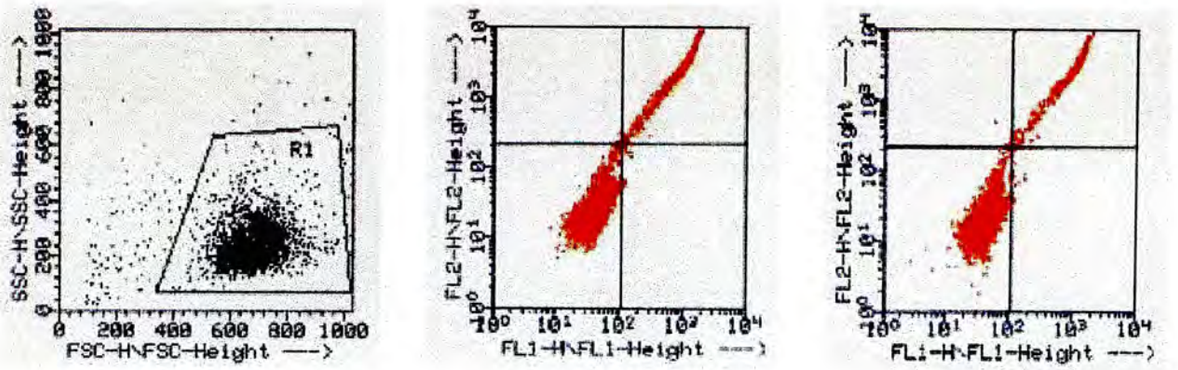


Figure 4.6 **Effects of FL and SCF on the Fold Increase of CFU-MK**

$CD34^+$ cells were cultured in either FL or SCF with TPO, IL-3 and IL-6 for 21 days. CFU-MK assay was performed by planting 3×10^3 cells per ml onto methylcellulose medium. Fold increases of CFU-MK in FL cultures was significantly higher than that in SCF cultures at day 14. Results were presented as mean ± S.E. n = 7, * $p < 0.05$.



(A) (B) (C)

Figure 4.7 **Flow Analysis of Expanded Cells: Determination of the Percentage of Megakaryocytic $CD61^+CD41^+$ Cells**

Expanded cells were stained with CD61-FITC (FL1) and CD41-PE (FL2) monoclonal antibodies. (B) represented FL-expanded cells and (C) represented SCF-expanded cells. Cell debris were gated out in their FSC-SSC dot plots (A). $CD61^+CD41^+$ cells were presented in the upper right quadrant.

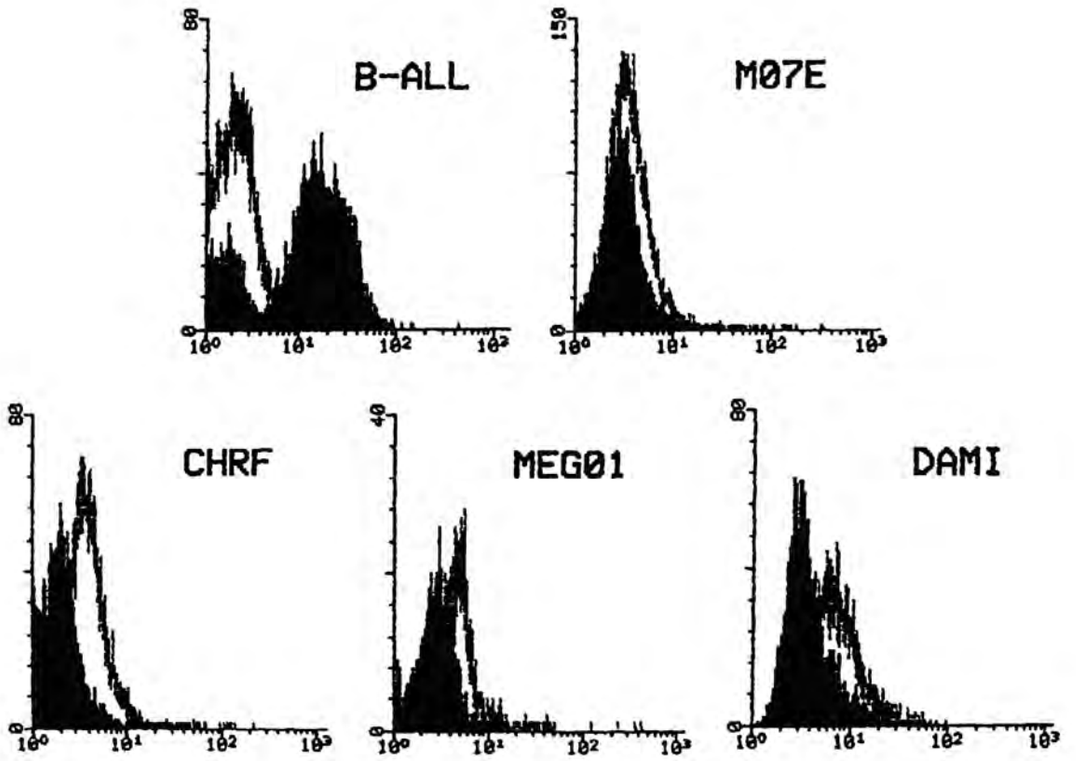


Figure 4.8 Flow Cytometry Analysis of Flt-3 Ligand Receptor on Megakaryocytes

Four human megkaryoblastic cell lines, M-07e, CHRF, Meg-01 and Dami, were stained with PE-conjugated anti-human FL receptor antibody. A human B-cell ALL sample was analyzed as a positive control of the antibody. Dark areas of histograms represented samples stained with the antibody and open areas represented samples stained with isotypic control. Results showed that the four cell lines did not express the receptor.

CHAPTER FIVE

EFFECTS OF MANNOSE-BINDING LECTIN ON THE *EX VIVO* EXPANSION OF HEMATOPOIETIC STEM AND PROGENITOR CELLS

Section 5.1 Results

5.1.1 *Ex Vivo* Expansion of $CD34^+$ Cells with Mannose-Binding Lectin

To study the effects of MBL on the *ex vivo* expansion of CB hematopoietic stem and progenitor cells, enriched $CD34^+$ cells at 2×10^4/ml were cultured in IMDM supplemented with 10% FCS. The cultures were performed in the presence of two cytokine treatments with the addition of various concentrations (0 – 800 ng/ml) of MBL for 35 days. Cytokines added to group A were 50 ng/ml TPO, 25 ng/ml SCF, 20 ng/ml IL-1β, 25 ng/ml IL-6 and 25 ng/ml IL-11. Cytokines added to group B were 50 ng/ml TPO, 25 ng/ml SCF and 50 ng/ml FL.

At day 0, the purity of enriched $CD34^+$ cells was 91.6 ± 1.65% (range 85.2 – 98.7%). The viability as determined by trypan blue dye exclusion assay were 93.4 ± 1.13% (range 87.5 – 96.0%), 87.5 ± 1.12% (range 82.2 – 90.4%), 78.3 ± 1.53% (range 70.8 – 83.5%) and 73.5 ± 1.18% (range 69.5 – 81.5%) at days 14, 21, 28 and 35, respectively. No significant difference in cell viability was observed between treatments. The expansion results at days 14, 21, 28 and 35 were summarized in Table 5.1 – 5.4. Total nucleated cells increased with the duration of culture. In both groups A and B, no significant difference was observed in the expansion of total nucleated cells with the addition of MBL (Figure 5.1).

Expansion of $CD34^+$ Cells

At day 14, the culture with 200 ng/ml MBL had the highest percentage of $CD34^+$ cells among all the group A cultures ($p < 0.05$) (Table 5.1). However, no significant difference was observed in the absolute numbers of $CD34^+$ cells. At days 21, 28 and 35, there were no significant differences in the percentages and the yields of $CD34^+$ cells between cultures with MBL and the control in group A although a trend of better expansion was observed in cultures with 200 ng/ml MBL (Table 5.2 – Table 5.4).

In group B, the percentages of $CD34^+$ cells in cultures with 200 ng/ml MBL were significantly higher than others at days 14, 21, 28 and 35 ($p < 0.05$) (Table 5.1 – 5.4). The yields of these cells were also the highest in cultures with 200 ng/ml MBL at days 21, 28 and 35. The fold increase of $CD34^+$ cells in culture with 200 ng/ml MBL at day 28 was significantly higher than that of the control ($p < 0.05$). In contrast, higher concentrations (400 and 800 ng/ml) of MBL decreased the expansion of these cells (Figure 5.2). At days 28 and 35, cultures with 800 ng/ml MBL showed significantly lower fold increases of $CD34^+$ cells when compared to the control cultures ($p < 0.05$). The fold increases of $CD34^+$ cells were the highest at day 28 (Figure 5.2). Comparing two cytokine treatments, the yields of $CD34^+$ cells in group B were significantly higher than those in group A at day 14, 28 and 35 ($p < 0.05$).

Expansion of $CD34^+CD38^-$ Cells

In group A, the percentage of $CD34^+CD38^-$ cells was significantly higher in cultures with 200 ng/ml MBL when compared to the respective control cultures at days 14 and 35 ($p < 0.05$) (Table 5.1 and Table 5.4). At days 21 and 28, no significant

difference in the percentage of these cells was observed among all the treatments (Table 5.2 and 5.3). Higher concentration of MBL again appeared to suppress the expansion of $CD34^+CD38^-$ cells. The fold increases of $CD34^+CD38^-$ cells in cultures with 800 ng/ml MBL were significantly lower than those of the control cultures at days 28 and 35 ($p < 0.05$) (Figure 5.3). The expansion of $CD34^+CD38^-$ cells was the highest at day 14.

In group B, the percentages and fold increases of $CD34^+CD38^-$ cells in cultures with 200 ng/ml MBL were superior to other cultures at all the time points ($p < 0.05$) (Table 5.1 – 5.4 and Figure 5.3 – 5.4). In addition, significantly higher percentages of these cells were observed in cultures with 100 ng/ml MBL when compared to the controls at days 14 and 28 ($p < 0.05$). Again, high concentrations of MBL appeared to suppress the expansion of $CD34^+CD38^-$ cells with significant decreases observed in cultures with 400 ng/ml MBL at day 28 and 800 ng/ml MBL at days 28 and 35 ($p < 0.05$) (Figure 5.3). The cytokines in group B cultures supported more efficient expansion of these cells than those in group A ($p < 0.05$ at days 14, 21, 28 and 35).

Expansion of Colony-Forming Unit

In group A, the yields of total CFU (CFU-GM + BFU/CFU-E + CFU-GEMM) in cultures with 200 ng/ml MBL were significantly higher than the control cultures at days 28 and 35 ($p < 0.05$) (Figure 5.5). The yield of total CFU was also higher in culture with 100 ng/ml MBL at day 35 when compared to the control ($p < 0.05$). In group B, 200 ng/ml MBL supported higher yields of total CFU when compared to the controls at days 14, 28 and 35 ($p < 0.05$). The yield of total CFU in culture with 800 ng/ml MBL was significantly lower than that of the control at day 35 ($p < 0.05$).

No significant differences were observed among two cytokine treatment groups and the expansion of total CFU was the highest at day 35.

For the expansion of CFU-GM, 200 ng/ml MBL in group A enhanced the fold increase of CFU-GM at days 14, 28 and 35 ($p < 0.05$) (Figure 5.6 and Figure 5.7). At days 14, 21 and 28, 800 ng/ml MBL inhibited the expansion of CFU-GM ($p < 0.05$). At day 35, significantly higher yield of CFU-GM was also observed in culture with 100 ng/ml MBL when compared to the control ($p < 0.05$). In group B, the cultures with 200 ng/ml MBL had the highest yield of CFU-GM at days 14, 28 and 35 ($p < 0.05$) (Table 5.1 – 5.4). Again, 800 ng/ml MBL inhibited the expansion at day 35 (Figure 5.6). Similar to total CFU, no significant differences were observed between groups A and B and the expansion of CFU-GM was the highest at day 35.

In group A, MBL appeared to have no effect on the erythroid lineage (Figure 5.8). The fold increases of BFU/CFU-E in group B were significantly higher than those in group A at day 14 and 21 ($p < 0.05$). In group B, 200 ng/ml MBL supported the highest yields of BFU/CFU-E at all the time points ($p < 0.05$) (Figure 5.7 and Figure 5.8). The expansion of BFU/CFU-E was the highest at day 14 and the lowest at day 35.

For the early progenitors, no significant difference in the expansion of CFU-GEMM was observed in group A (Figure 5.9). In contrast, 200 ng/ml MBL supported the highest yields and fold increases of CFU-GEMM in group B at all the time points ($p < 0.05$) (Table 5.1 – 5.4, Figure 5.7 and Figure 5.9). Similarly to other CFU subsets, 400 and 800 ng/ml MBL produced less CFU-GEMM than the controls ($p < 0.05$ at

day 14). The expansion of CFU-GEMM was significantly higher in group B when compared to those in group A at days 14, 21 and 28. In addition, the yields of CFU-GEMM decreased with time.

Considering the megakaryocytic lineage, MBL did not exert any effect on the expansion of $CD61^+CD41^+$ cells in both groups A and B (Table 5.1 – 5.4). No significant difference was found in the expansion of CFU-MK in both groups except at day 14 in group B. The fold increase of CFU-MK with 200 ng/ml MBL at day 14 was the highest among all cultures in both groups A and B ($p < 0.05$) (Figure 5.10).

5.1.2 Effects of Mannose-Binding Lectin on the Preservation of Early Stem and Progenitor Cells

Total CFU

To examine the effects of MBL on the preservation of hematopoietic stem and progenitor cells, MNC at 2×10^5/ml or enriched $CD34^+$ cells at 2×10^4/ml were cultured in X-Vivo 10 without any cytokine supplement in the presence of 0 – 1,600 ng/ml MBL for 35 days. For comparison, some cultures contained 40 ng/ml FL with or without 200 ng/ml MBL. High concentrations of MBL appeared to suppress the formation of total CFU at 400 – 1,600 ng/ml while 200 ng/ml MBL exerted similar effects as FL and FL+MBL200 (Figure 5.11). At day 14, significantly higher total CFU formations were also observed in MNC cultures with FL and FL+MBL200 when compared to those of the control ($p < 0.05$). No significant difference was seen when 200 ng/ml MBL was compared to the control (Table 5.5). At days 21, 28 and 35, the yields of total CFU in cultures with 200 ng/ml MBL, FL and FL+MBL200 were higher than those of the respective control cultures ($p < 0.05$) (Table 5.6 – 5.8).

Significant lower yields of total CFU were observed in cultures with 800 and 1,600 ng/ml MBL at days 14, 28 and 35 when compared to the control cultures. Among all time points, no significant difference was found when 200 ng/ml MBL was added to cultures with FL (Figure 5.11).

In $CD34^+$ cell cultures, 200 ng/ml MBL and FL+MBL200 produced higher yields of total CFU than the control at day 14 ($p < 0.05$). On the other hand, lower yields of total CFU were observed in cultures with 400 – 1,600 ng/ml MBL when compared to the control at day 14 ($p < 0.05$) (Table 5.5). At days 21, 28 and 35, similar significant results were observed in cultures with both MNC and $CD34^+$ cells (Figure 5.11).

CFU-GM

In MNC cultures, FL and FL+MBL200 produced significantly higher yields of CFU-GM than the respective controls at days 14 and 21 while 400 – 1,600 ng/ml MBL produced lower yields (Figure 5.12 and Table 5.5 – 5.6). At day 28, significantly higher yields of CFU-GM were observed in cultures with 100 ng/ml and 200 ng/ml MBL, FL and FL+MBL200 ($p < 0.05$). Again, high concentrations (800 and 1,600 ng/ml) of MBL suppressed the expansion (Table 5.7). At day 35, 200 ng/ml MBL, FL and FL+MBL200 produced higher yields of CFU-GM than the control ($p < 0.05$) (Table 5.8).

In $CD34^+$ cell cultures, 200 ng/ml MBL and FL+MBL200 produced higher yields of CFU-GM than the control at day 14 while 400 – 1,600 ng/ml MBL suppressed the expansion ($p < 0.05$) (Figure 5.12 and Table 5.5). At day 21, more efficient expansion was observed in cultures with 200 ng/ml MBL, FL as well as

FL+MBL200 ($p < 0.05$) (Figure 5.12 and Table 5.6). No significant difference was observed at day 28 between cultures with MBL and the control (Table 5.7). Again, 200 ng/ml MBL, FL and FL+MBL200 produced higher yields of CFU-GM than the control at day 35 while the yield in culture with 1,600 ng/ml MBL was lower ($p < 0.05$) (Table 5.8). Moreover, the yields of CFU-GM in cultures with 200 ng/ml MBL and FL+MBL200 were significantly higher than that with FL alone ($p < 0.05$) (Figure 5.12).

BFU/CFU-E

In MNC cultures, the yields of BFU/CFU-E in cultures with FL and FL+MBL200 were significantly higher than those of the respective control cultures at all time points ($p < 0.05$) (Figure 5.13). At days 28 and 35, 200 ng/ml MBL supported higher yields of BFU/CFU-E ($p < 0.05$) (Table 5.7 and Table 5.8).

In cultures with $CD34^+$ cells, similar effects of MBL and FL were seen (Figure 5.13). In addition, MBL at high concentrations (400 – 1,600 ng/ml) produced less BFU/CFU-E than the control cultures (Figure 5.13).

CFU-GEMM

For the early progenitors, 200 ng/ml MBL, FL and FL+MBL200 supported higher production of CFU-GEMM in MNC cultures (Figure 5.14). The yield of CFU-GEMM in cultures with FL+MBL200 was significantly higher than that with FL alone at day 21 ($p < 0.05$) (Table 5.6).

In $CD34^+$ cell cultures, 200 ng/ml MBL was shown to support the highest yields of

CFU-GEMM (Figure 5.14). At day 14, 100 and 200 ng/ml MBL, FL and FL+MBL200 produced more CFU-GEMM than the control ($p < 0.05$). Significant difference was also observed with the addition of 200 ng/ml MBL to the culture with FL (Table 5.5). At days 21 and 28, the yields of CFU-GEMM in cultures with 100 and 200 ng/ml MBL, FL and FL+MBL were significantly higher than those of the control cultures ($p < 0.05$) (Table 5.6 and Table 5.7). At day 35, 200 ng/ml MBL, FL and FL+MBL200 supported significantly higher yields of CFU-GEMM than the control. The addition of 200 ng/ml MBL to the culture with FL significantly increased the yields of CFU-GEMM ($p < 0.05$) (Figure 5.14 and Table 5.8).

5.1.3 Transplantation of Expanded Cells into NOD/SCID Mice

Enriched $CD34^+$ cells were cultured in IMDM + 10% FCS with TPO, SCF and FL in the presence or absence of 200 ng/ml MBL for 14 days. Expanded cells from nine CB samples were then transplanted into 50 NOD/SCID mice, 25 for each group. Five mice from each group died within 6 weeks of transplantation. In the group without MBL, 15 out of 19 mice showed engraftment of expanded human cells as analyzed by flow cytometry (Figure 5.15 – 5.18) and PCR analysis (Figure 5.19). The percentage of human $CD45^+$ cells in the BM of transplanted mice was 16.7 ± 5.47% (range 0.01 – 71.0%) (Figure 5.15 and Figure 5.19). The percentages of human $CD45^+$ cells in the spleen (Figure 5.16) and PB (Figure 5.17) of mice were 2.97 ± 1.19% (range 0.00 – 12.9%) and 0.83 ± 0.50% (range 0.00 – 6.70%), respectively (Figure 5.19). For those mice with more than 1% human $CD45^+$ cells in the BM, the hematopoietic subsets were analyzed by flow cytometry (Figure 5.18). The percentages of human $CD45^+$ subpopulations $CD34^+$, $CD33^+$, $CD14^+$, $CD19^+$ and $CD61^+$ cells were 1.28 ± 0.41% (range 0.05 – 3.14%), 30.1 ± 6.50% (range 16.8 –

47.2%), 8.08 ± 1.53% (range 4.91 – 12.3%), 12.3 ± 2.51 (range 4.43 – 17.9%) and 0.32 ± 0.27% (range 0.00 – 1.40%), respectively.

In the group of mice transplanted with MBL-expanded cells, 17 out of 19 mice showed engraftment of expanded human cells. The percentages of human $CD45^+$ cells in the BM, spleen and PB of transplanted mice measured by flow cytometry were 11.4 ± 4.39% (range 0.04 – 79.3%), 1.29 ± 0.64% (range 0.00 – 6.60%) and 0.73 ± 0.29% (range 0.01 – 3.26%), respectively (Figure 5.19). The human $CD45^+$ cells in BM consisted of 1.59 ± 0.29% (range 0.44 – 3.20%) $CD34^+$ cells, 24.2 ± 6.83% (range 13.3 – 51.1%) $CD33^+$ cells, 7.60 ± 2.60% (range 2.96 – 17.6%) $CD14^+$ cells, 19.8 ± 6.83% (range 0.00 – 42.14%) $CD19^+$ cells and 0.30 ± 0.23% (range 0.00 – 1.18%) $CD61^+$ cells (Figure 5.20), Paired t-test or Wilcoxon Sign Rank test, whichever suitable, was performed to compare the engrafting ability of cells expanded in both groups. No significant difference was observed.

Section 5.2 Discussion

The aim of this study was to elucidate the effects of a plant MBL on hematopoietic stem and progenitor cells. Several approaches were applied, including 1) *ex vivo* expansion of CB $CD34^+$ cells, 2) preservation of primitive hematopoietic stem and progenitor cells and 3) transplantation of MBL-expanded cells into NOD/SCID mice.

Two cytokine cocktails were selected for the expansion of megakaryocytic progenitors (group A containing TPO, IL-1β, IL-6 and IL-11) and early progenitor cells (group B containing TPO, SCF and FL). In general, there was a trend of superior expansion of megakaryocytic cells in group A, with up to 15.9% of

$CD61^+CD41^+$ cells at day 14. The early progenitor cells in terms of $CD34^+CD38^-$ cells and CFU-GEMM were significantly higher in group B.

MBL had no effect on the cell viability and expansion of nucleated cells and megakaryocytic lineage ($CD61^+CD41^+$ cells and CFU-MK) at all doses and time points. On the other hand, MBL at 200 ng/ml significantly increased the expansion of early progenitor cells including the populations of $CD34^+$ cells, $CD34^+CD38^-$ cells and CFU-GEMM up to 32.2-fold, 282-fold and 27.5-fold, respectively in the presence of three early-acting cytokines TPO, SCF and FL. However, increasing dosage of MBL to 400 and 800 ng/ml had inhibitory effects on the expansion.

The ability of MBL on the preservation of early stem and progenitor cells was studied in a serum-free X-Vivo 10 medium without cytokine supplements for 35 days. Using MNC and enriched $CD34^+$ cells as the seeding populations, CFU of all lineages significantly decreased with the duration of culture. MBL at 0 – 200 ng/ml preserved the CFU at a dose-dependent manner but the effect declined with the increase in the concentration of MBL. The addition of FL at 40 ng/ml also significantly preserved these progenitors. Interestingly, MBL had a more tremendous effect than FL on the expansion of CFU-GEMM at days 14 and 35, and CFU-GM at day 35. The addition of MBL to cultures with FL, however, did not enhance the effect of FL.

We further compared the engraftment capacity of progenitor cells expanded with group B cytokines for 14 days with and without MBL in sub-lethally irradiated NOD/SCID mice. Although the majority of transplanted mice showed engraftment of

human $CD45^+$ cells and subsets in their BM, spleen and PB, the engraftment was not affected by the presence of MBL in the expansion.

Our results appeared to agree with those of Colucci *et al.* (1999) who reported that flt-3 receptor-interacting lectin (FRIL) extracted from red kidney beans preserved CFU from CB MNC in X-Vivo 10 for one month without medium change. Kollet *et al.* (2000) then reported that CB $CD34^+$ cells pre-incubated with FRIL alone could respond to cytokine stimulation and engrafted in NOD/SCID mice. They also reported that over 90% of cells after 13 days of incubation with FRIL were in the resting phase of the cell cycle.

Our study demonstrated that MBL at 200 ng/ml had the ability to preserve early stem and progenitor cells in *in vitro* cultures but higher dosage appeared to have adverse effects. However, the mechanism of such action of this plant MBL is unclear. Human serum MBL has been shown to play an important role in immune defence, particularly during the phase of primary contact with microorganisms through its binding specificity to the repeating sugar arrays on microbial surfaces (Turner *et al.* 1998). In our study, the MBL purified from plant materials act differently from other cytokines such as FL which preserves early progenitors but concurrently promotes both proliferation and differentiation (Dooley *et al.* 1997, Haylock *et al.* 1997, Abkowitz *et al.* 1998, Goff *et al.* 1998). One reason why we did not observe any effect of MBL on the NOD/SCID mouse transplantation was possibly because the experimental design was inadequate, considering that cultures at day 14 with or without MBL might contain sufficient SCID-repopulating cells (SRC) for engraftment. In addition, a larger sample size and a longer duration post-transplant

might be required to demonstrate quantitative differences between the two treatment groups.

In conclusion, MBL at 200 ng/ml appeared to enhance the expansion and preservation of early hematopoietic stem and progenitors. Future investigations are needed to confirm the mechanism of this agent. If proven to be effective, MBL could be applied for the *ex vivo* expansion of hematopoietic stem and progenitor cells.

Table 5.1 – 5.4 **Effects of Mannose-Binding Lectin on the *Ex Vivo* Expansion of Cord Blood $CD34^+$ Cells at Day 14, 21, 28 and 35**

Enriched $CD34^+$ cells at 2×10^4/ml were cultured in IMDM + 10% FCS with two cytokine treatments plus various concentrations of MBL for 35 days. Tables (A) were cell products cultured with 50 ng/ml TPO, 25 ng/ml SCF, 20 ng/ml IL-1β, 25 ng/ml IL-6 and 20 ng/ml IL-11. Tables (B) were cell products cultured with 50 ng/ml TPO, 25 ng/ml SCF and 50 ng/ml FL. MBL was added to each culture at 0, 50, 100, 200, 400 and 800 ng/ml. Cells from 1ml of cultures at day 0 were harvested for analysis at days 14, 21, 28 and 35. Results were expressed as mean ± S.E. n = 8, * $p < 0.05$ (higher than the control) and $^+ p < 0.05$ (lower than the control).

Table 5.1 Effects of Mannose-Binding Lectin on the *Ex Vivo* Expansion of Cord Blood $CD34^+$ Cells at Day 14

(A)

	Control	MBL50	MBL100	MBL200	MBL400	MBL800
TNC ($\times 10^6$)	2.98 ± 1.03	2.45 ± 0.40	1.80 ± 0.30	1.89 ± 0.26	2.39 ± 0.24	2.08 ± 0.31
$CD34^+$ Cells (%)	6.21 ± 1.34	5.77 ± 0.89	5.67 ± 0.97	8.10 ± 0.88*	5.19 ± 0.85	5.06 ± 0.86
$CD34^+$ Cells ($\times 10^5$)	1.85 ± 0.05	1.41 ±0.03	1.02 ±0.03	1.53 ± 0.03	1.24 ± 0.02	1.05 ± 0.03
$CD34^+CD38^-$ Cells (%)	0.47 ± 0.24	0.85 ± 0.30	0.93 ± 0.33	1.22 ± 0.43	0.70 ± 0.25	0.46 ± 0.10
$CD34^+$ $CD38^-$ Cells ($\times 10^4$)	1.59 ± 8.10	2.00 ±0.73	1.73 ± 6.60	2.14 ± 9.50	1.45 ± 5.60	0.65 ± 3.50$^+$
Total CFU ($\times 10^4$)	4.89 ± 0.76	4.54 ± 0.82	3.99 ± 0.83	6.46 ± 1.15	3.27 ± 0.64	2.47 ± 0.56
CFU-GM ($\times 10^4$)	3.93 ± 1.65	3.68 ± 1.37	3.00 ± 1.16	5.44 ± 3.65*	2.37 ± 1.77	1.67 ± 1.05$^+$
BFU/CFU-E ($\times 10^3$)	6.40 ± 3.52	5.37 ± 2.58	6.55 ± 1.70	6.65 ± 3.73	5.81 ± 1.20	4.95 ± 3.38
CFU-GEMM ($\times 10^3$)	3.22 ± 1.17	3.28 ± 2.45	3.37 ± 1.85	3.55 ± 2.54	3.17 ± 2.15	3.05 ± 1.52
$CD61^+CD41^+$ Cells (%)	14.9 ± 3.13	15.9 ± 2.61	11.9 ± 2.67	12.0 ± 1.78	15.0 ± 1.58	12.1 ± 2.03
$CD61^+CD41^+$ Cells ($\times 10^5$)	4.36 ± 1.54	3.69 ± 1.01	2.30 ± 0.94	2.16 ± 0.44	3.51 ± 0.55	2.83 ± 1.09
CFU-MK ($\times 10^6$)	1.32 ± 0.55	1.65 ± 1.03	2.01 ± 0.88	2.13 ± 0.85	1.88 ± 0.71	1.65 ± 0.98

(B)

	Control	MBL50	MBL100	MBL200	MBL400	MBL800
TNC ($\times 10^6$)	2.91 ± 0.35	2.97 ± 0.32	2.98 ± 0.38	2.82 ± 0.37	2.79 ± 0.50	2.77 ± 0.21
$CD34^+$ Cells (%)	7.68 ± 0.49	7.50 ± 0.68	8.00 ± 0.57	8.76 ± 0.57*	7.32 ± 0.48	6.33 ± 0.44
$CD34^+$ Cells ($\times 10^5$)	2.28 ± 0.36	2.21 ± 0.30	2.37 ± 0.35	2.47 ± 0.32	1.98 ± 0.32	1.79 ± 0.23
$CD34^+CD38^-$ Cells (%)	2.13 ± 0.20	2.32 ± 0.24	2.53 ± 0.27*	3.32 ± 0.19*	2.61 ± 0.18	1.84 ± 0.20
$CD34^+$ $CD38^-$ Cells ($\times 10^4$)	6.09 ± 0.88	6.88 ± 1.07	7.24 ± 1.02	9.43 ± 1.35*	6.75 ± 0.72	5.11 ± 0.70
Total CFU ($\times 10^4$)	3.99± 0.92	4.57 ± 1.23	5.81 ± 1.82	8.34 ± 2.12*	4.58 ± 2.39	3.38 ± 0.57
CFU-GM ($\times 10^4$)	2.39 ± 1.17	2.97 ± 3.50	3.95 ± 4.50	6.08 ± 4.40*	3.04 ± 1.15	1.87 ± 0.88
BFU/CFU-E ($\times 10^3$)	10.1 ± 2.55	9.77 ± 3.88	11.2 ± 5.00	13.2 ± 3.00*	9.98 ± 3.20	9.50 ± 5.21
CFU-GEMM ($\times 10^3$)	5.90 ± 1.18	6.23 ± 4.58	7.45 ± 1.64	9.42 ± 2.65*	5.44 ± 3.94	5.56 ± 4.00
$CD61^+CD41^+$ Cells (%)	8.32 ± 1.31	7.96 ± 1.29	8.06 ± 1.12	7.50 ± 1.13	7.47 ± 1.45	5.98 ± 0.94
$CD61^+CD41^+$ Cells ($\times 10^5$)	2.31 ± 0.47	2.25 ± 0.42	2.27 ± 0.46	1.89 ± 0.36	1.63 ± 0.30	1.64 ± 0.26
CFU-MK ($\times 10^6$)	2.85 ± 0.87	3.06 ± 1.05	3.19 ± 0.68	3.95 ± 1.56	3.01 ± 2.17	2.98 ± 0.99

Table 5.2 Effects of Mannose-Binding Lectin on the *Ex Vivo* Expansion of Cord Blood $CD34^+$ Cells at Day 21

(A)

	Control	MBL50	MBL100	MBL200	MBL400	MBL800
TNC ($\times 10^6$)	8.58 ± 1.30	8.58 ± 2.19	7.69 ± 2.31	7.89 ± 2.71	10.3 ± 1.96	8.44 ± 1.31
$CD34^+$ Cells (%)	1.98 ± 0.69	1.87 ± 0.52	1.96 ± 0.55	3.41 ± 0.57	1.70 ± 0.32	1.59 ± 0.41
$CD34^+$ Cells ($\times 10^5$)	1.69 ± 0.10	1.60 ±0.11	1.51 ± 0.13	2.70 ± 0.16	1.75 ± 0.06	1.34 ± 0.06
$CD34^+CD38^-$ Cells (%)	0.03 ± 0.01	0.06 ± 0.02	0.06 ± 0.02	0.10 ± 0.02	0.04 ± 0.01	0.02 ± 0.01
$CD34^+$ $CD38^-$ Cells ($\times 10^4$)	2.47 ± 0.85	4.12 ± 1.24	3.13 ± 0.45	5.02 ± 1.19	3.73 ± 1.00	1.28 ± 1.29
Total CFU ($\times 10^4$)	8.95 ± 2.24	7.60 ± 1.32	7.23 ± 2.08	9.13 ± 3.00	7.84 ± 1.29	5.37 ± 0.95
CFU-GM ($\times 10^5$)	8.21 ± 3.42	6.96 ± 2.65	6.49 ± 3.33	8.34 ± 5.12	7.20 ± 5.71	4.75 ± 1.16$^+$
BFU/CFU-E ($\times 10^5$)	5.40 ± 2.34	4.20 ± 1.16	5.18 ± 2.20	5.60 ± 2.11	4.54 ± 1.65	4.30 ± 0.85
CFU-GEMM ($\times 10^5$)	2.00 ± 0.98	2.18 ± 1.02	2.19 ± 1.07	2.28 ± 0.54	1.88 ± 0.78	1.92 ± 0.44
$CD61^+CD41^+$ Cells (%)	0.91 ± 0.14	0.92 ± 0.15	0.81 ± 0.17	1.16 ± 0.19	0.94 ± 0.17	0.50 ± 0.13
$CD61^+CD41^+$ Cells ($\times 10^5$)	2.77 ± 1.16	2.15 ± 1.32	4.13 ± 1.46	5.85 ± 2.07	4.11 ± 1.45	2.15 ± 0.76
CFU-MK ($\times 10^5$)	9.45 ± 3.28	9.65 ± 4.55	11.1 ± 5.21	13.0 ± 2.55	7.54 ± 1.42	8.84 ± 2.52

(B)

	Control	MBL50	MBL100	MBL200	MBL400	MBL800
TNC ($\times 10^6$)	6.12 ± 0.34	5.75 ± 0.39	5.46 ± 0.40	5.52 ± 0.60	5.02 ± 0.46	5.47 ± 0.31
$CD34^+$ Cells (%)	4.89 ± 0.44	4.81 ± 0.30	5.03 ± 0.38	6.09 ± 0.35*	4.42 ± 0.31	4.13 ± 0.10
$CD34^+$ Cells ($\times 10^5$)	3.07 ± 0.39	2.80 ± 0.31	2.85 ± 0.39	3.49 ± 0.52*	2.27 ±0.32	2.27 ± 1.63
$CD34^+CD38^-$ Cells (%)	1.15 ± 0.15	1.54 ± 0.22	1.56 ± 0.15	2.29 ± 0.14*	1.32 ± 0.17	0.93 ± 0.16
$CD34^+$ $CD38^-$ Cells ($\times 10^4$)	6.95 ± 0.90	8.56 ± 1.03	8.42 ± 1.00	12.5 ± 1.29*	6.68 ± 1.04	5.11 ± 0.97
Total CFU ($\times 10^4$)	4.77 ± 0.39	4.06 ± 0.40	4.31 ± 0.58	5.19 ± 0.44	3.97 ± 0.34	4.57 ± 1.00
CFU-GM ($\times 10^5$)	3.46 ± 2.10	2.65 ± 0.87	2.82 ± 0.45	3.37 ± 0.18	2.59 ± 1.12	3.26 ± 1.19
BFU/CFU-E ($\times 10^5$)	8.75 ± 4.32	9.23 ± 1.79	9.88 ± 3.28	11.5 ± 2.98*	9.11 ± 4.20	8.92 ± 3.24
CFU-GEMM ($\times 10^5$)	4.32 ± 1.78	4.88 ± 3.50	5.05 ± 2.46	6.73 ± 2.97*	4.73 ± 2.85	4.20 ± 3.50
$CD61^+CD41^+$ Cells (%)	1.48 ± 0.26	1.36 ± 0.33	1.34 ± 0.31	1.45 ± 0.33	1.24 ± 0.31	1.23 ± 0.34
$CD61^+CD41^+$ Cells ($\times 10^4$)	9.39 ± 1.91	7.98 ± 1.97	7.64 ± 2.11	8.03 ± 2.21	6.04 ± 1.51	7.28 ± 2.28
CFU-MK ($\times 10^5$)	11.6 ± 3.56	12.2 ± 4.32	13.3 ± 2.78	15.6 ± 1.88	12.8 ± 7.12	11.7 ± 3.16

Table 5.3 Effects of Mannose-Binding Lectin on the *Ex Vivo* Expansion of Cord Blood $CD34^+$ Cells at Day 28

(A)

	Control	MBL50	MBL100	MBL200	MBL400	MBL800
TNC ($\times 10^6$)	19.8 ± 3.96	15.2 ± 2.27	17.4 ± 3.88	16.2 ± 4.22	21.2 ± 5.02	16.6 ± 3.34
$CD34^+$ Cells (%)	1.35 ± 0.54	1.47 ±0.63	1.44 ± 0.65	2.07 ± 0.80	1.14 ± 0.51	0.99 ± 0.48
$CD34^+$ Cells ($\times 10^5$)	2.67 ±0.22	2.24 ± 0.14	2.50 ±0.25	3.35 ± 0.34	2.41 ± 0.26	1.65 ± 0.16
$CD34^+CD38^-$ Cells (%)	0.21 ± 0.13	0.23 ± 0.15	0.16 ± 0.07	0.20 ± 0.12	0.23 ± 0.12	0.13 ± 0.09
$CD34^+$ $CD38^-$ Cells ($\times 10^4$)	7.05 ± 4.81	5.14 ± 3.44	4.20 ± 1.53	11.3 ± 4.01	14.9 ± 5.27	7.54 ± 2.67
Total CFU ($\times 10^4$)	6.51 ± 0.81	6.54 ± 0.90	8.65 ± 1.56	13.4 ± 2.92*	8.82 ± 2.45	4.13 ± 0.80
CFU-GM ($\times 10^5$)	5.90 ± 0.98	5.88 ± 1.65	7.91 ± 3.28	12.6 ± 3.66*	8.16 ± 1.49	3.56 ± 1.33$^+$
BFU/CFU-E ($\times 10^5$)	4.90 ± 1.65	5.20 ± 2.65	5.98 ± 1.49	6.11 ± 1.65	5.42 ± 1.65	4.38 ± 1.42
CFU-GEMM ($\times 10^5$)	1.18 ± 0.16	1.39 ± 0.88	1.42 ± 0.59	1.66 ± 0.55	1.17 ± 0.46	1.28 ± 0.99
$CD61^+CD41^+$ Cells (%)	0.59 ± 0.15	0.43 ± 0.07	0.43 ± 0.09	1.20 ± 1.30	2.54 ± 0.15	2.51 ± 0.12
$CD61^+CD41^+$ Cells ($\times 10^5$)	1.82 ± 0.26	1.08 ± 0.71	0.85 ± 1.27	2.10 ± 1.66	1.26 ± 2.45	1.12 ± 1.45
CFU-MK ($\times 10^5$)	7.21 ± 2.65	7.32 ± 5.45	8.42 ± 1.24	8.55 ± 2.60	7.11 ± 2.45	6.98 ± 3.22

(B)

	Control	MBL50	MBL100	MBL200	MBL400	MBL800
TNC ($\times 10^6$)	12.4 ± 2.11	12.1 ± 1.59	11.8 ± 1.48	11.4 ± 1.64	11.0 ± 1.35	10.6 ± 0.98
$CD34^+$ Cells (%)	3.84 ± 0.45	4.39 ±0.30	4.44 ±0.39	5.48 ± 0.37*	3.75 ±0.32	3.17 ± 0.29
$CD34^+$ Cells ($\times 10^5$)	4.58 ± 0.68	5.23 ± 0.69	5.32 ± 0.80	6.24 ± 0.92*	4.29 ± 0.69	3.47 ± 0.51$^+$
$CD34^+CD38^-$ Cells (%)	1.49 ± 0.14	1.73 ± 0.28	1.81 ± 0.18*	2.54 ± 0.16*	1.64 ± 0.25	1.24 ±0.21
$CD34^+$ $CD38^-$ Cells ($\times 10^4$)	19.3 ± 4.18	19.9 ± 3.47	21.6 ± 5.54	29.0 ± 5.04*	18.1 ± 0.36$^+$	14.4 ± 3.42$^+$
Total CFU ($\times 10^4$)	4.34 ± 0.78	4.42 ± 0.91	6.38 ± 1.43	8.38 ± 1.56*	5.09 ± 1.41	6.15 ± 1.98
CFU-GM ($\times 10^5$)	3.58 ± 2.80	3.58 ± 3.88	5.40 ± 2.84	7.05 ± 2.79*	4.27 ± 2.17	5.36 ± 3.29
BFU/CFU-E ($\times 10^5$)	4.65 ± 1.88	5.32 ± 3.73	6.73 ± 3.22	8.42 ± 2.46*	5.14 ± 1.98	4.99 ± 2.06
CFU-GEMM ($\times 10^5$)	2.94 ± 0.95	3.05 ± 1.17	3.11 ± 1.83	4.87 ± 1.23*	3.02 ± 2.27	2.96 ± 1.65
$CD61^+CD41^+$ Cells (%)	0.45 ± 0.09	0.50 ±0.14	0.47 ±0.17	0.53 ±0.13	0.52 ± 0.18	0.32 ± 0.07
$CD61^+CD41^+$ Cells ($\times 10^4$)	5.31 ± 1.58	5.59 ± 1.35	4.72 ±1.27	5.19 ± 1.12	4.65 ± 1.01	3.48 ± 0.89
CFU-MK ($\times 10^5$)	8.65 ± 3.22	9.11 ± 1.49	9.65 ± 3.78	9.99 ± 5.85	8.99 ± 2.71	8.74 ± 1.36

Table 5.4 Effects of Mannose-Binding Lectin on the *Ex Vivo* Expansion of Cord Blood $CD34^+$ Cells at Day 35

(A)

	Control	MBL50	MBL100	MBL200	MBL400	MBL800
TNC ($\times 10^6$)	21.8 ±2.57	21.2 ±3.53	29.9 ± 4.33	28.6 ± 4.25	26.5 ± 6.87	24.5 ± 2.25
$CD34^+$ Cells (%)	0.24 ± 0.13	0.25 ± 0.13	0.28 ± 0.14	0.39 ± 0.18	0.09 ±0.03	0.02 ± 0.01
$CD34^+$ Cells ($\times 10^5$)	0.52 ± 0.04	0.53 ± 0.05	0.83 ± 0.06	1.11 ± 0.08	0.23 ± 0.02	0.49 ± 0.02
$CD34^+CD38^-$ Cells (%)	0.05 ± 0.03	0.07 ± 0.04	0.10 ± 0.05	0.17 ± 0.19*	0.04 ± 0.02	0.02 ± 0.01
$CD34^+$ $CD38^-$ Cells ($\times 10^4$)	0.84 ± 4.22	1.14 ± 5.30	2.71 ± 13.8	4.26 ± 20.3	0.81 ± 2.37	0.23 ± 1.36$^+$
Total CFU ($\times 10^4$)	13.4 ± 1.54	10.6 ± 2.01	21.6 ± 4.83*	30.1 ± 6.73*	19.1 ± 4.82	23.1 ± 1.01
CFU-GM ($\times 10^5$)	13.0 ± 5.46	10.0 ± 5.16	21.0 ± 3.53*	29.5 ± 15.7*	18.7 ± 5.16	22.7 ± 3.09
BFU/CFU-E ($\times 10^5$)	3.20 ± 1.49	4.81 ± 1.47	4.53 ± 2.58	4.99 ± 1.69	3.38 ± 1.99	3.11 ± 1.65
CFU-GEMM ($\times 10^5$)	0.97 ± 0.16	1.01 ± 0.69	1.11 ± 1.05	1.24 ± 0.50	0.95 ± 0.45	0.88 ± 0.23
$CD61^+CD41^+$ Cells (%)	0.05 ± 0.01	0.06 ± 0.01	0.12 ± 0.04	0.51 ±0.14	0.07 ± 0.02	0.27 ± 0.13
$CD61^+CD41^+$ Cells ($\times 10^4$)	1.40 ± 0.50	1.22 ± 0.14	1.79 ± 0.53	1.91 ± 0.19	1.15 ± 0.03	0.99 ± 0.08
CFU-MK ($\times 10^5$)	5.65 ± 1.13	5.45 ± 1.24	4.21 ± 1.60	6.00 ± 1.88	5.42 ± 1.62	5.12 ± 1.32

(B)

	Control	MBL50	MBL100	MBL200	MBL400	MBL800
TNC ($\times 10^6$)	19.9 ± 1.47	19.6 ± 1.66	19.3 ±1.95	19.7 ± 1.59	18.2 ± 1.35	16.0 ± 1.62
$CD34^+$ Cells (%)	2.00 ± 0.12	1.84 ± 0.09	1.97 ±0.04	2.52 ± 0.13*	1.84 ± 0.14	1.48 ± 0.08
$CD34^+$ Cells ($\times 10^5$)	4.49 ± 0.65	4.08 ±0.58	4.19 ± 0.66	4.71 ± 0.58*	3.27 ± 0.59$^+$	2.41 ± 0.40$^+$
$CD34^+CD38^-$ Cells (%)	1.11 ± 0.18	0.85 ± 0.20	0.99 ± 0.16	1.29 ± 0.23*	0.98 ± 0.23	0.74 ± 0.21
$CD34^+$ $CD38^-$ Cells ($\times 10^4$)	25.2 ± 0.47	19.2 ± 0.52	21.9 ± 0.58	24.9 ± 0.61*	18.1 ± 0.54	12.2 ± 0.36$^+$
Total CFU ($\times 10^4$)	14.1 ± 2.14	14.4 ± 2.33	17.6 ± 3.20	24.8 ± 2.82*	16.8 ± 3.88	9.09 ± 0.94$^+$
CFU-GM ($\times 10^5$)	13.7 ± 5.98	13.9 ± 4.96	17.0 ± 6.83	24.1 ± 8.68*	16.3 ± 8.42	8.70 ± 1.19$^+$
BFU/CFU-E ($\times 10^5$)	3.17 ± 1.54	3.85 ± 1.49	4.01 ± 1.73	4.99 ± 2.41*	3.45 ± 1.65	3.00 ± 1.93
CFU-GEMM ($\times 10^5$)	1.02 ± 0.85	1.11 ± 0.25	1.55 ± 0.46	2.46 ± 0.98*	1.13 ± 0.08	0.95 ± 0.07
$CD61^+CD41^+$ Cells (%)	0.05 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.12 ± 0.02	0.06 ± 0.01	0.05 ± 0.02
$CD61^+CD41^+$ Cells ($\times 10^4$)	1.07 ± 0.29	1.36 ± 0.36	0.30 ± 0.38	2.37 ± 0.50	1.10 ± 0.20	0.66 ± 0.23
CFU-MK ($\times 10^5$)	3.56 ± 1.94	4.22 ± 2.87	6.78 ± 3.65	8.88 ± 2.98	7.21 ± 3.56	6.13 ± 2.78

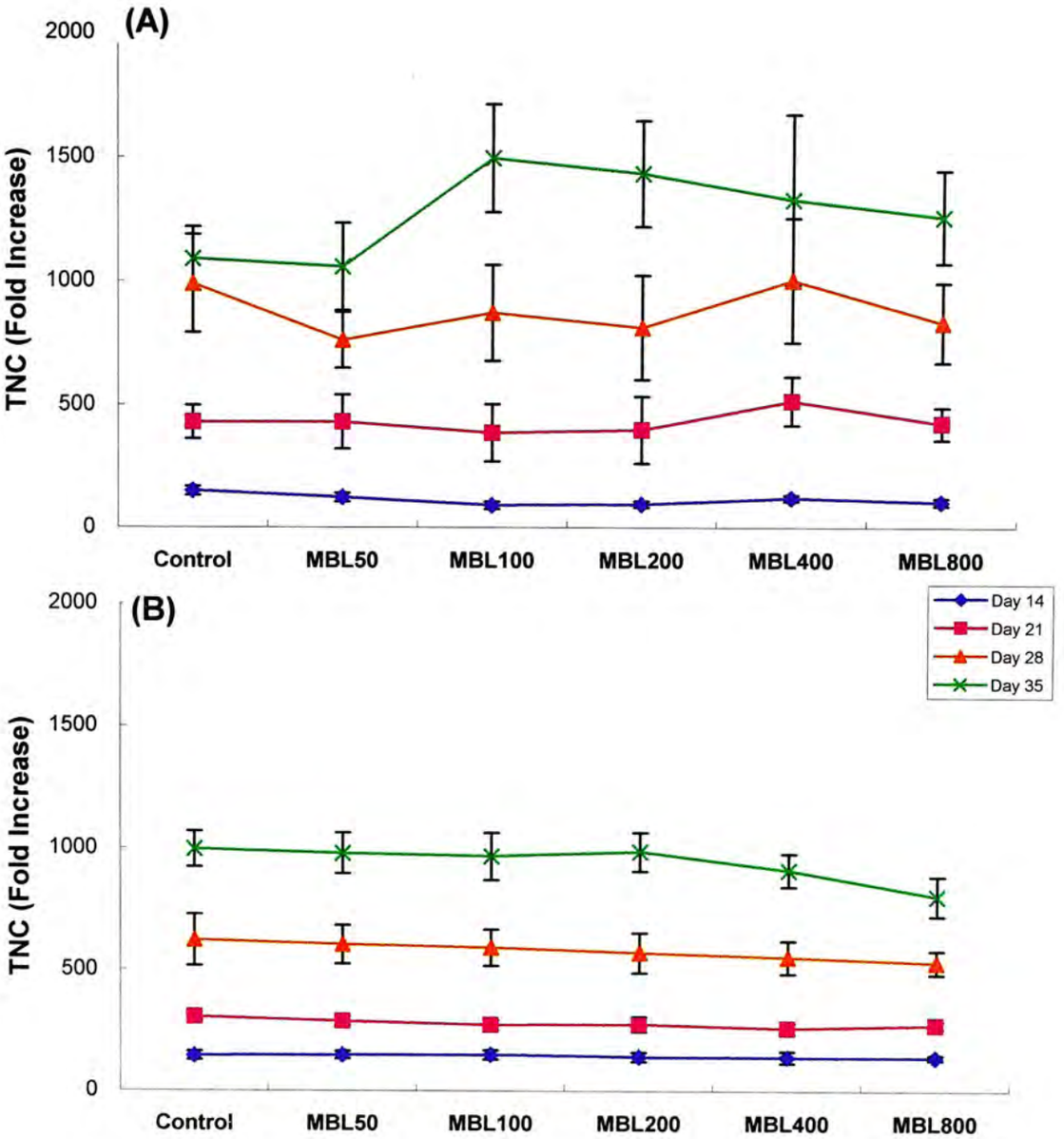


Figure 5.1 **Effects of Mannose-Binding Lectin on the *Ex Vivo* Expansion of Total Nucleated Cells**

MBL had no effect on the fold increases of total nucleated cells with both cytokine treatments. (A) – 50 ng/ml TPO, 25 ng/ml SCF, 20 ng/ml IL-1β, 25 ng/ml IL-6 and 25 ng/ml IL-11. (B) – 50 ng/ml TPO, 25 ng/ml SCF and 50 ng/ml FL. Results were expressed mean ± S.E. n= 8

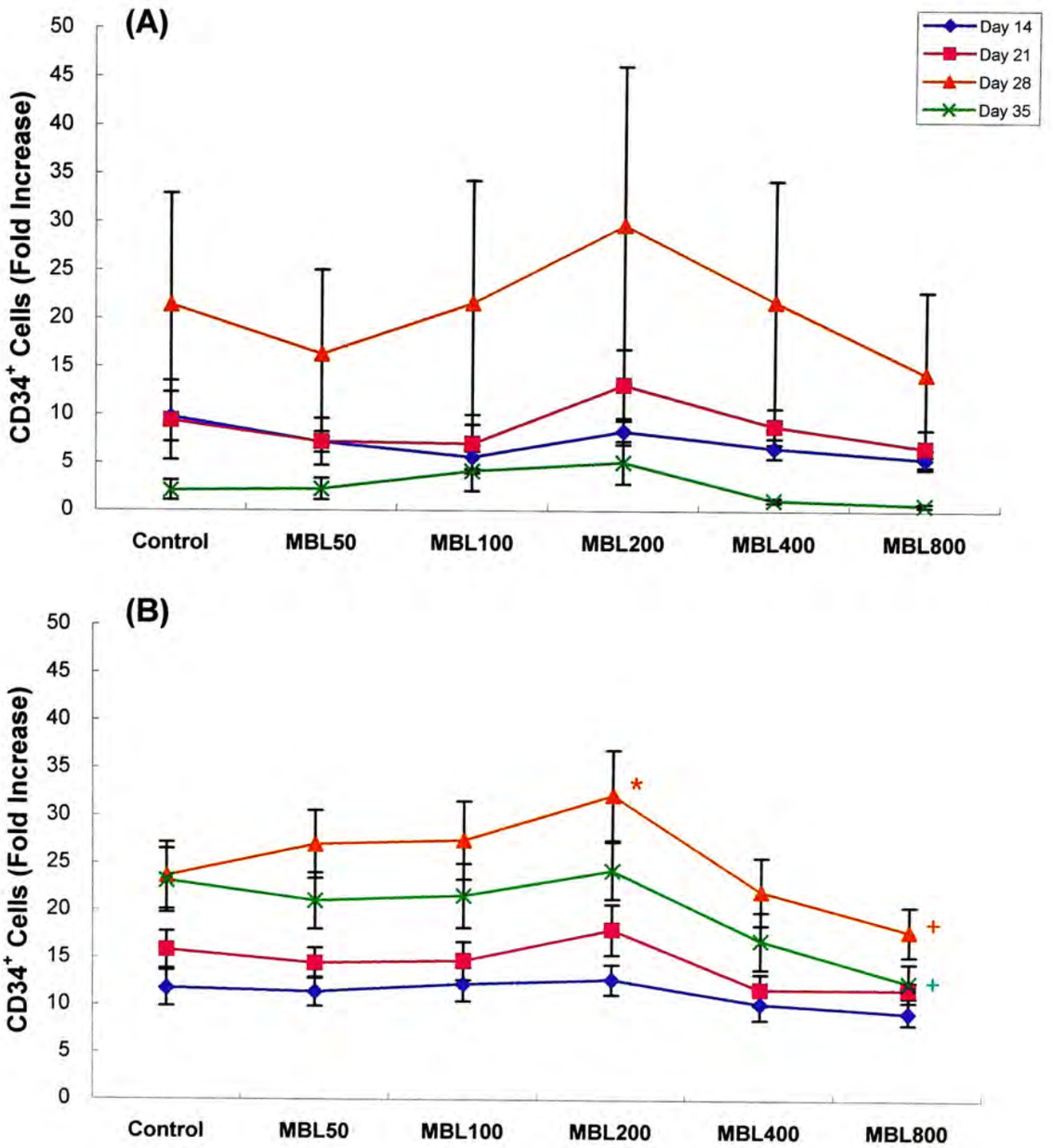


Figure 5.2 **Effects of Mannose-Binding Lectin on the *Ex Vivo* Expansion of $CD34^+$ Cells**

MBL supported more efficient expansion of $CD34^+$ cells at 200 ng/ml while 800 ng/ml was found to suppress these cells. * and $^+$ represented that significant differences in the fold increases of $CD34^+$ cells when compared to the control cultures ($p < 0.05$). Results were expressed mean ± S.E. n= 8

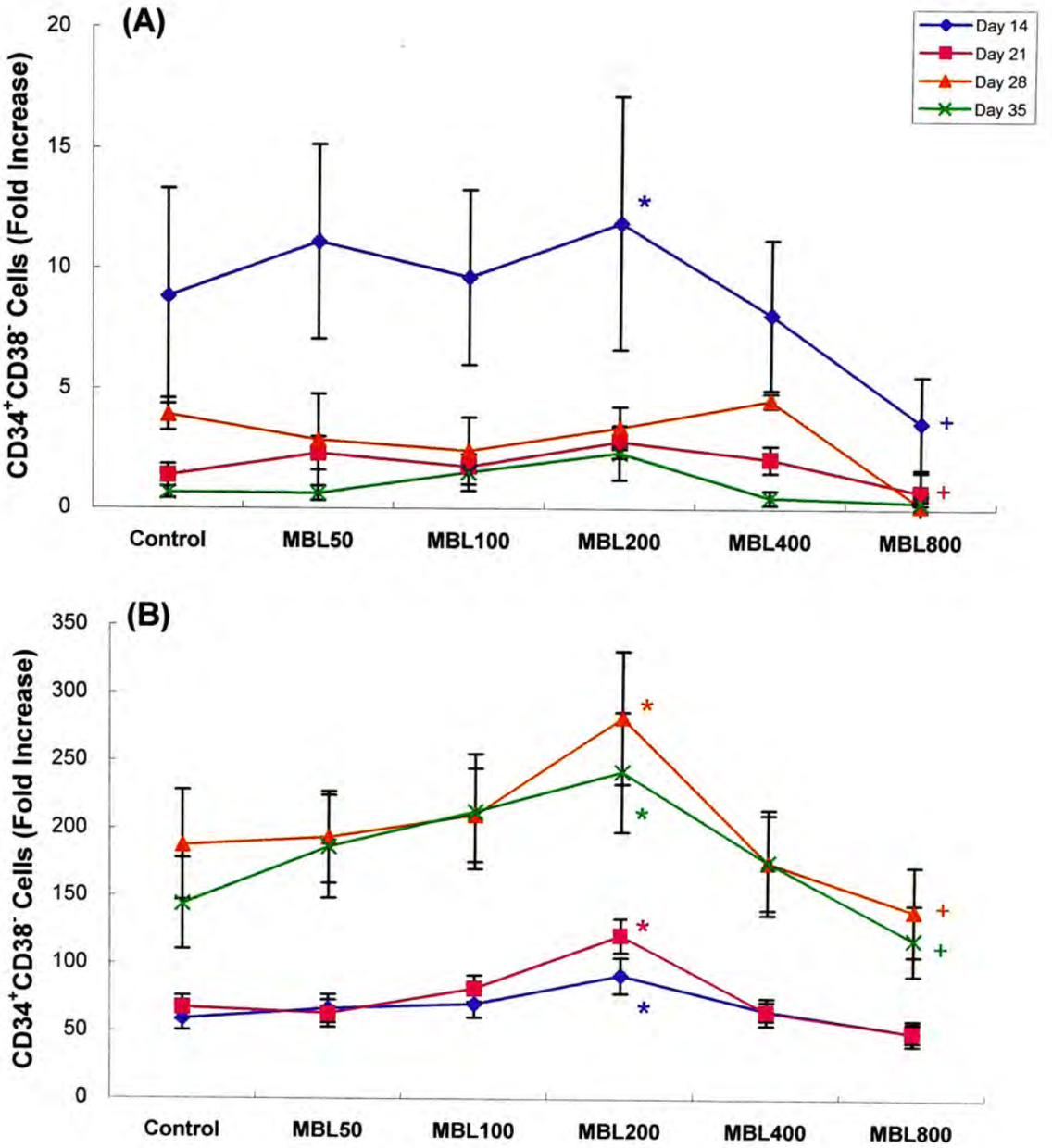


Figure 5.3 **Effects of Mannose-Binding Lectin on the *Ex Vivo* Expansion of $CD34^+CD38^-$ Cells**

Similar to $CD34^+$ cells, *significantly higher fold increases of $CD34^+CD38^-$ cells were shown in cultures with 200 ng/ml MBL and $^+$significantly lower fold increases were seen in cultures with 800 ng/ml MBL when compared to control cultures ($p < 0.05$). Results were expressed mean ± S.E. n= 8

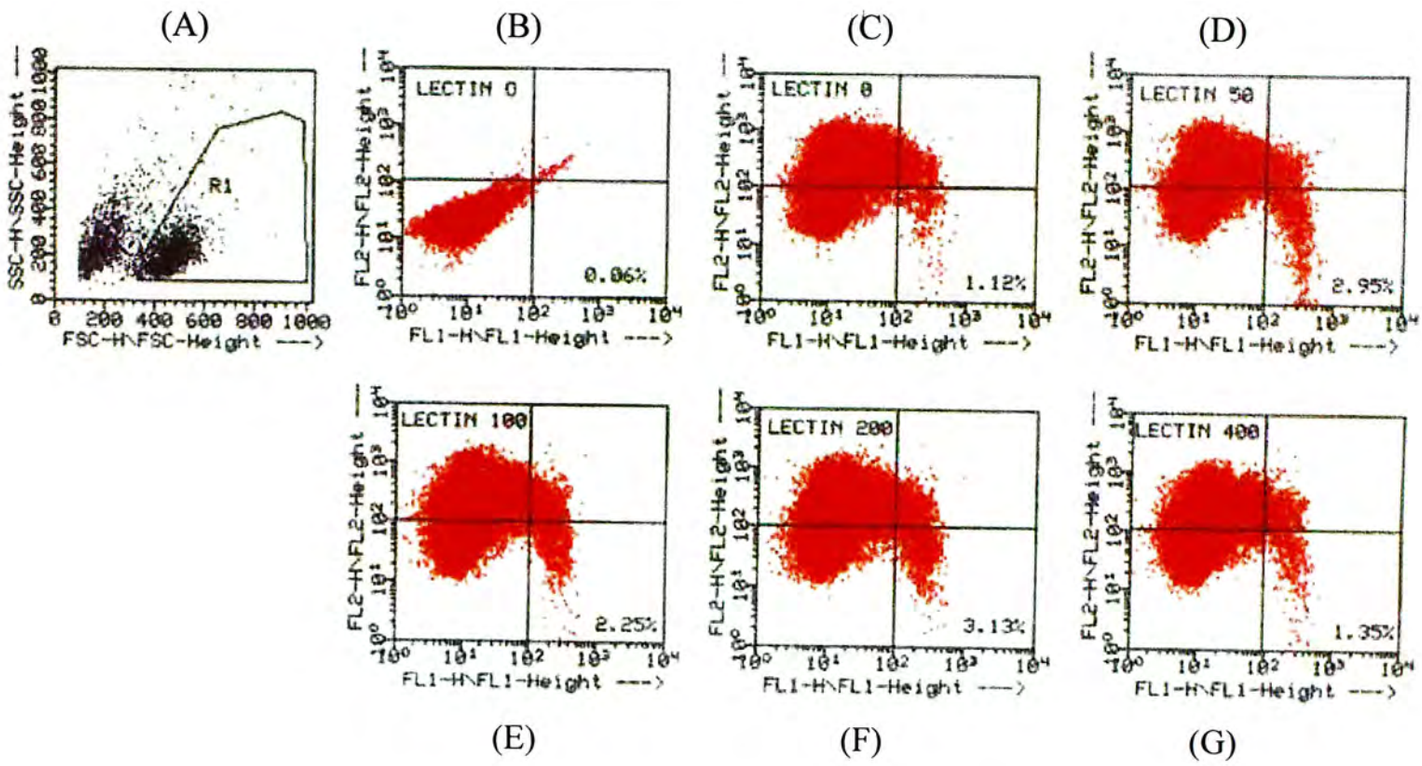


Figure 5.4 **Flow Cytometry Analysis of $CD34^+$ Cells and $CD34^+CD38^-$ Cells**

Expanded cells were harvested at days 14, 21, 28 and 35 for flow cytometry analysis. Cells expanded with 0 – 400 ng/ml MBL were stained with CD34-FITC and CD38-PE monoclonal antibodies (C – G) and their isotypic controls (B). Results showed that 200 ng/ml MBL enhanced higher percentage of $CD34^+CD38^-$ cells after 14 days of culture (F).

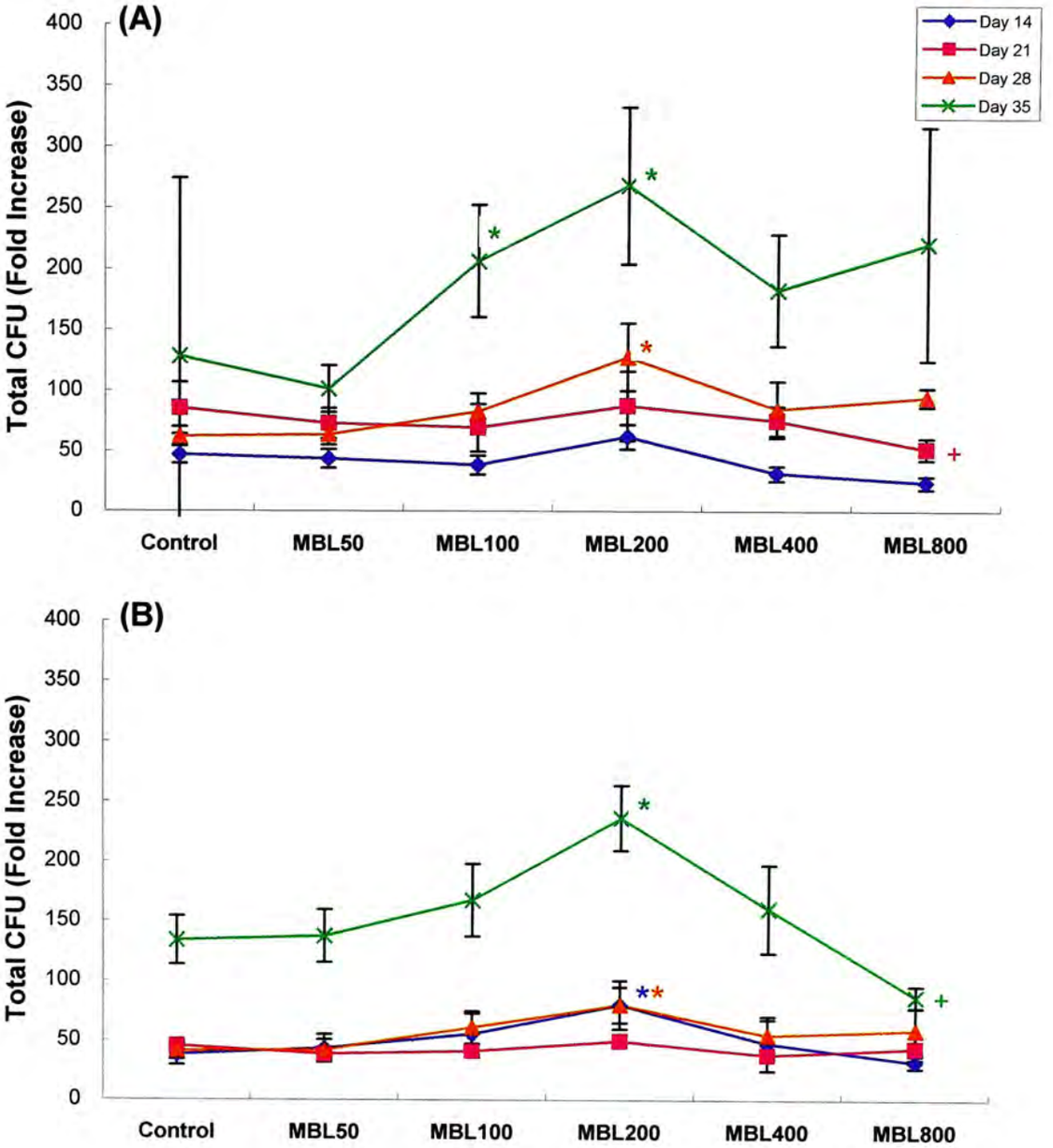


Figure 5.5 **Effects of Mannose-Binding Lectin on the Fold Increase of Total CFU after *Ex Vivo* Expansion**

Expanded cells were harvested for CFU assay at days 14, 21, 28 and 35 of culture. MBL *increased the expansion of CFU at lower concentrations (100 – 200 ng/ml) but $^{+}$inhibited the expansion at 800 ng/ml ($p < 0.05$) when compared to the control cultures. Results were expressed mean ± S.E. n= 8

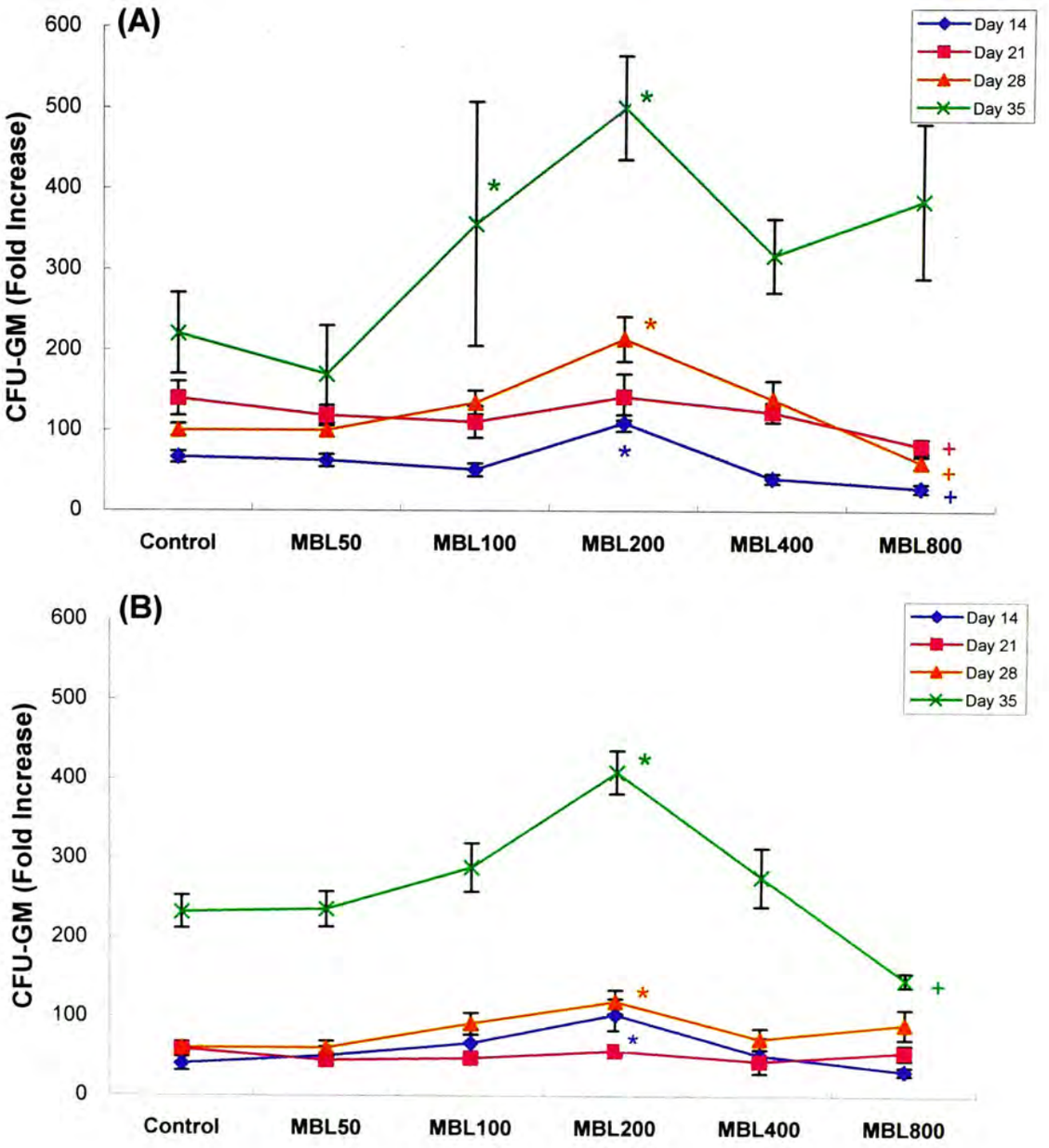
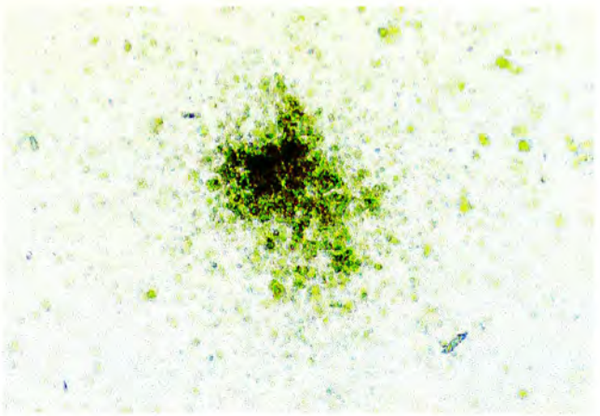


Figure 5.6 **Effects of Mannose-Binding Lectin on the Fold Increase of CFU-GM after *Ex Vivo* Expansion**

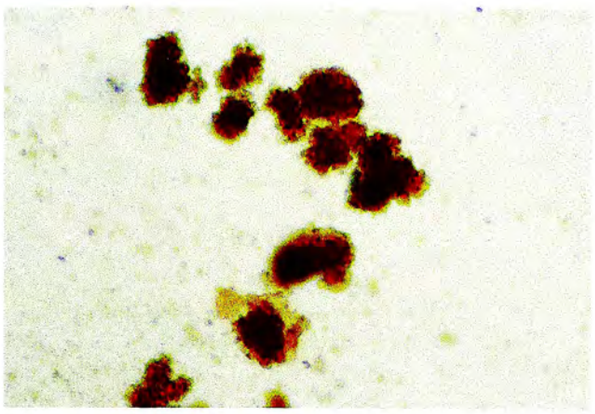
Fold Increases of CFU-GM in both groups A and B peaked at day 35. The fold increases were the highest in cultures with 200 ng/ml MBL (* $p < 0.05$). Again, 800 ng/ml MBL decreased the fold increases of CFU-GM when compared to the control (+ $p < 0.05$). Results were expressed mean ± S.E. n= 8

(A)



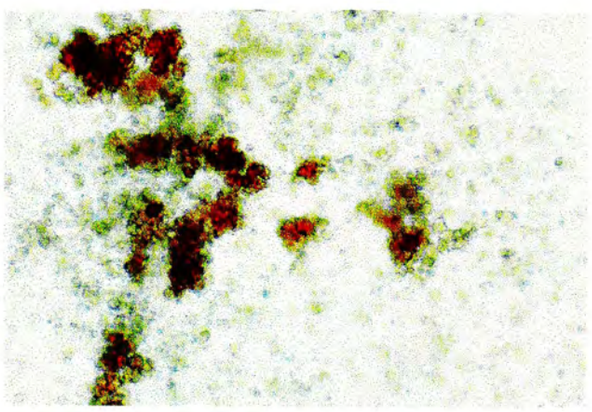
50×

(B)



50×

(C)



50×

Figure 5.7 CFU derived from MBL-Expanded Cells

(A) CFU-GM, (B) BFU-E and (C) CFU-GEMM were derived from cells expanded with 50 ng/ml TPO, 25 ng/ml SCF, 50 ng/ml FL and 200 ng/ml MBL for 35 days.

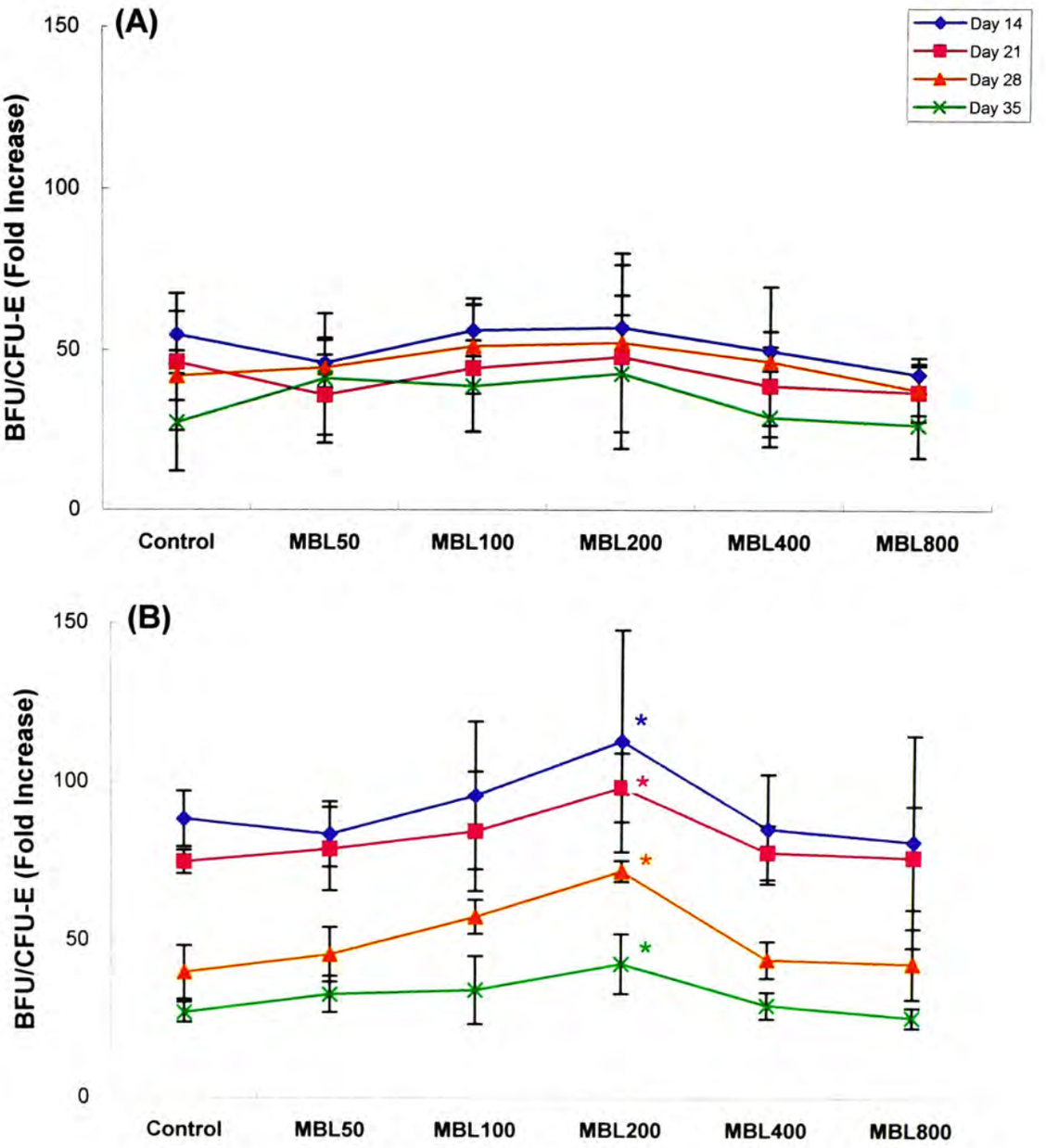


Figure 5.8 **Effects of Mannose-Binding Lectin on the Fold Increase of BFU/CFU-E after *Ex Vivo* Expansion**

No significant difference was observed in fold increases of BFU/CFU-E in group A. The fold increases in cultures with 200 ng/ml MBL were higher than those of the control in group B (* $p < 0.05$). Results were expressed mean ± S.E. n= 8

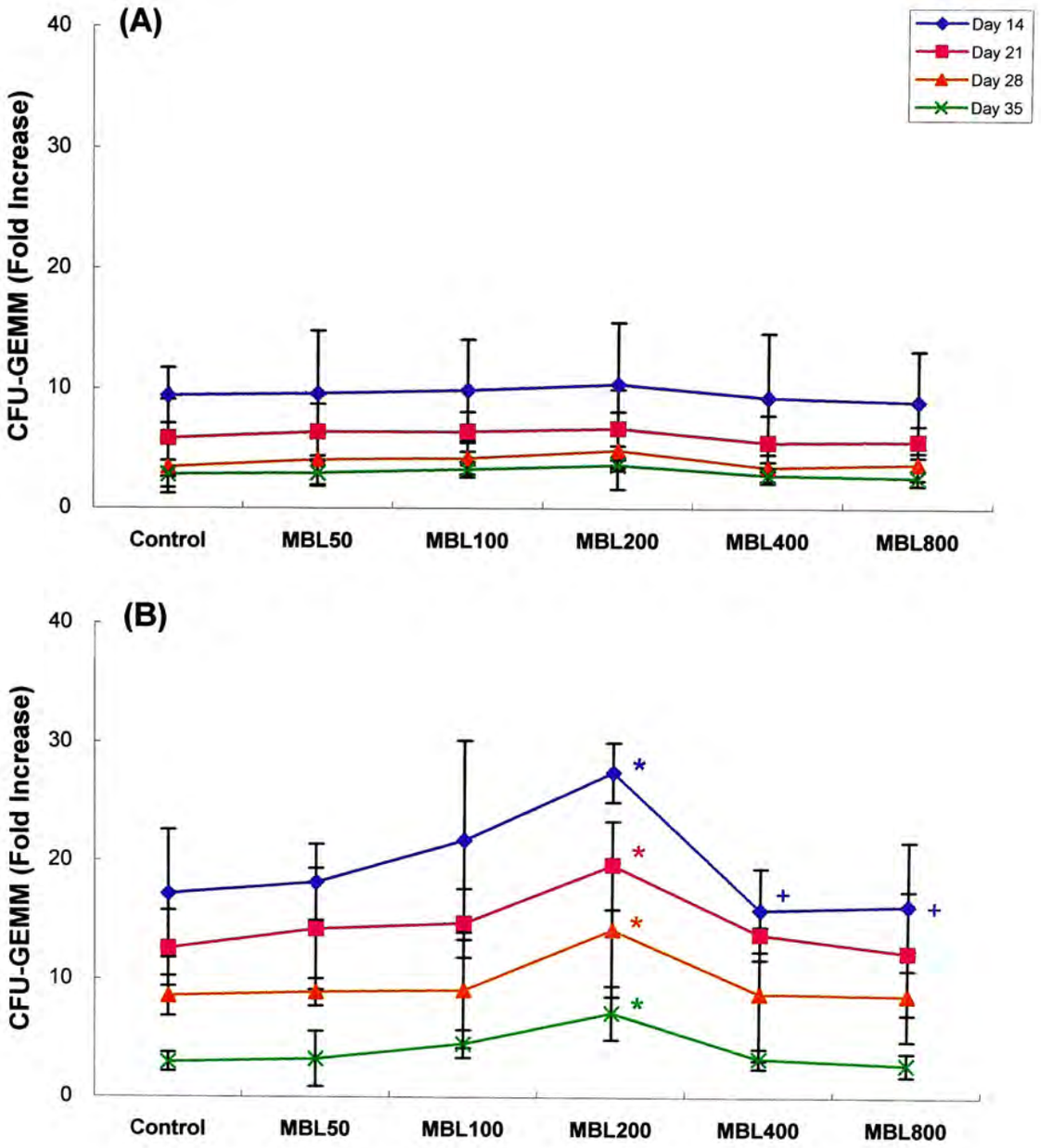


Figure 5.9 **Effects of Mannose-Binding Lectin on the Fold Increase of CFU-GEMM after *Ex Vivo* Expansion**

No significant difference was observed in fold increases of CFU-GEMM in (A). In (B), *200 ng/ml MBL enhanced the expansion of CFU-GEMM while $^{+}$400 and 800 ng/ml suppressed the expansion at day 14 ($p < 0.05$) when compared to the control cultures. Results were expressed mean ± S.E. n= 8

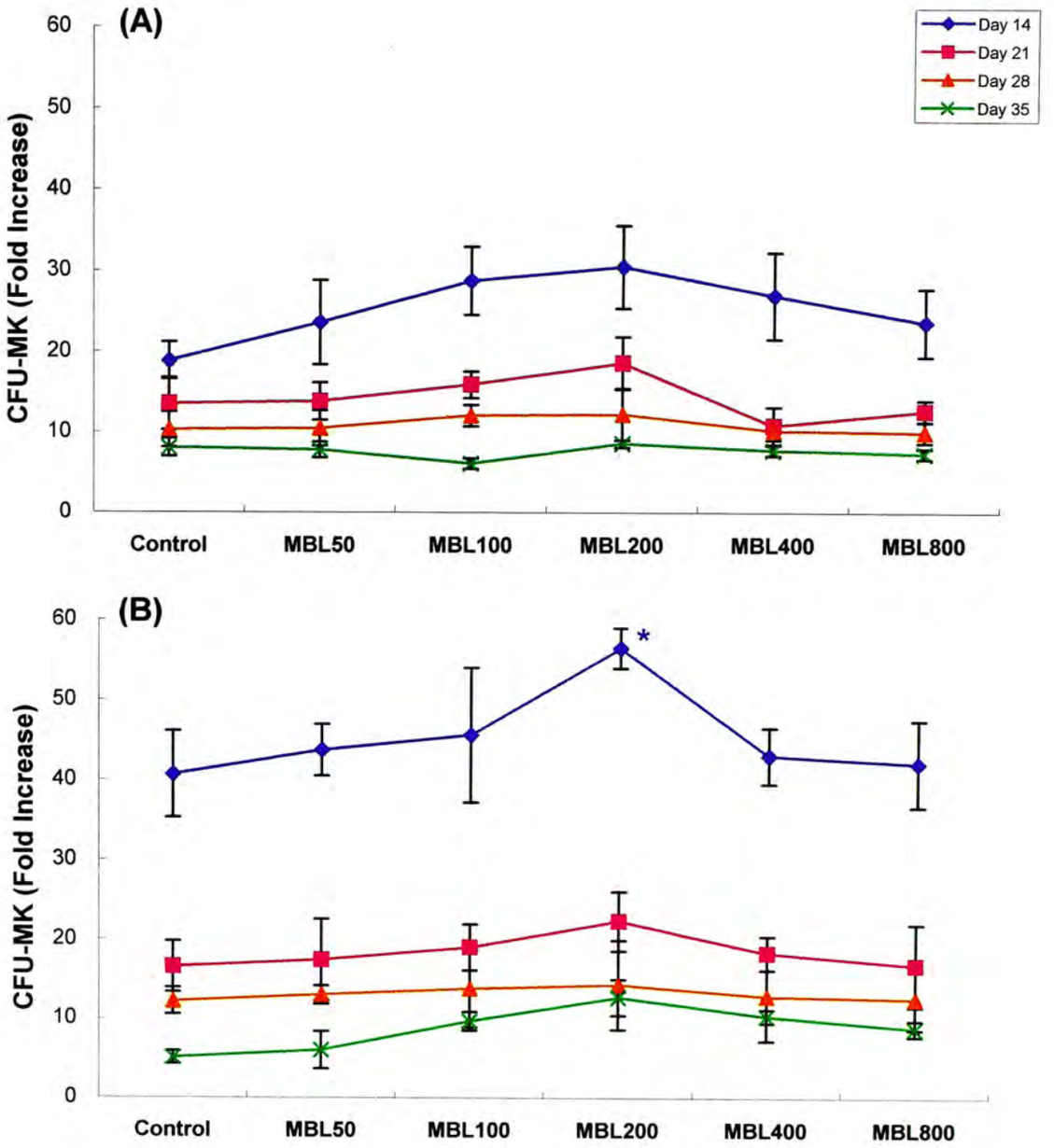


Figure 5.10 Effects of Mannose-Binding Lectin on the Fold Increase of CFU-MK after *Ex Vivo* Expansion

The formation of CFU-MK decreased with duration of culture. No significant differences were found between treatments except the fold increase of CFU-MK which was higher with 200 ng/ml MBL than the control in group B (* $p < 0.05$). Results were expressed mean ± S.E. n= 8

Table 5.5 – 5.8 **Effects of Mannose-Binding Lectin on CFU Formation at Day 14, 21, 28 and 35**

(A) MNC at 2×10^5/ml or (B) $CD34^+$ cells at 2×10^4/ml were cultured in X-Vivo 10 with 0 – 1,600 ng/ml MBL, 40 ng/ml FL or 40 ng/ml FL + 200 ng/ml MBL for 35 days. Results were presented as the mean number of CFU formed from 5 ml cultures at day 0 ± S.E. n = 8, * $p < 0.05$ (higher than the control) and $^+$ $p < 0.05$ (lower than the control).

Table 5.5 Effects of Mannose-Binding Lectin on the CFU Formation at Day 14

(A)

	Control	MBL100	MBL200	MBL400	MBL800	MBL1600	FL	FL+MBL200
Total CFU	460 ± 20.7	384 ± 16.1	491 ± 19.6	334 ± 17.0	299 ± 43.5^{+}	227 ± 56.4 $^{+}$	570 ± 18.7 *	620 ± 18.8 *
CFU-GM	321 ± 30.5	304 ± 13.5	355 ± 35.5	230 ± 22.9^{+}	229 ± 29.0^{+}	173 ± 33.0^{+}	442 ± 42.0*	442 ± 24.5*
BFU/CFU-E	90.5 ± 9.00	56.5 ± 6.00	87.0 ± 7.32	70.5 ± 10.1	41.5 ± 9.87	40.2 ± 23.2	113 ± 12.0*	126 ± 11.1*
CFU-GEMM	24.0 ± 4.00	48.8 ± 7.80	65.2 ± 5.11 *	34.3 ± 10.0	30.1 ± 5.00	30.4 ± 6.32	69.7 ± 17.1*	33.5 ± 11.9

(B)

	Control	MBL100	MBL200	MBL400	MBL800	MBL1600	FL	FL+MBL200
Total CFU	610 ± 26.3	600 ± 28.4	735 ± 33.3*	394 ± 7.87^{+}	336 ± 6.72^{+}	334 ± 6.67^{+}	640 ± 44.5	730 ± 39.5*
CFU-GM	314 ± 13.5	375 ± 44.5	406 ± 16.0*	197 ± 16.5^{+}	192 ± 42.3^{+}	209 ± 44.4^{+}	338 ± 36.0	376 ± 32.0*
BFU/CFU-E	197 ± 26.0	154 ± 24.2	229 ± 28.8	98.5 ± 18.1^{+}	62.5 ± 10.0^{+}	83.5 ± 13.5^{+}	213 ± 53.2	238 ± 38.1
CFU-GEMM	39.7 ± 14.3	99.1 ± 28.8*	132 ± 13.2*	77.1 ± 18.4	48.0 ± 7.90	46.7 ± 6.42	88.5 ± 8.71*	119 ± 29.8*

Table 5.6 Effects of Mannose-Binding Lectin on the CFU Formation at Day 21

(A)

	Control	MBL100	MBL200	MBL400	MBL800	MBL1600	FL	FL+MBL200
Total CFU	225 ± 20.8	325 ± 25.8	465 ± 32.4*	237 ± 63.2	167 ± 10.6	154 ± 2.87	458 ± 30.9*	453 ± 19.8*
CFU-GM	266 ± 65.5	259 ± 48.2	329 ± 25.9*	160 ± 15.6^{+}	184 ± 14.4^{+}	107 ± 11.6^{+}	326 ± 16.8*	291 ± 11.9*
BFU/CFU-E	56.5 ± 6.5	38.5 ± 8.11	75.5 ± 6.42	55.5 ± 6.32	24.0 ± 5.51	44.3 ± 4.18	81.0 ± 31.8*	70.0 ± 8.11
CFU-GEMM	14.2 ± 2.98	29.2 ± 9.83	56.7 ± 6.15*	30.0 ± 11.2	0.00 ± 0.00	0.00 ± 0.00	29.5 ± 5.97	60.0 ± 5.88*

(B)

	Control	MBL100	MBL200	MBL400	MBL800	MBL1600	FL	FL+MBL200
Total CFU	350 ± 10.5	414 ± 36.8	492 ± 26.7*	292 ± 58.3^{+}	315 ± 63.3^{+}	175 ± 35.0^{+}	499 ± 41.1	505 ± 35.7*
CFU-GM	156 ± 10.5	200 ± 45.5	238 ± 23.5*	167 ± 46.5	145 ± 35.7	140 ± 44.4	236 ± 36.0*	292 ± 32.0*
BFU/CFU-E	91.5 ± 11.2	108 ± 18.5	154 ± 15.9*	83.5 ± 13.3	45.2 ± 5.09^{+}	0.00 ± 0.00^{+}	167 ± 16.2*	109 ± 28.1
CFU-GEMM	37.9 ± 17.0	72.0 ± 22.0*	77.5 ± 11.8*	41.7 ± 13.2	45.0 ± 5.55	35.0 ±3.78	80.5 ± 7.42*	76.5 ± 16.0*

Table 5.7 Effects of Mannose-Binding Lectin on the CFU Formation at Day 28

(A)

	Control	MBL100	MBL200	MBL400	MBL800	MBL1600	FL	FL+MBL200
Total CFU	192 ± 14.5	262 ± 17.4	308 ± 15.6*	172 ± 1.56	92.5 ± 6.93^{+}	96.5 ± 9.90^{+}	297 ± 17.2*	336 ± 20.9*
CFU-GM	169 ± 38.5	220 ± 21.2*	218 ± 18.5*	146 ± 16.5	92.5 ± 22.5^{+}	86.5 ± 26.5^{+}	226 ± 35.5*	256 ± 15.6*
BFU/CFU-E	22.6 ± 3.60	38.8 ± 9.11	64.5 ± 4.98*	26.3 ± 12.2	0.00 ± 0.00	10.0 ± 2.50	57.0 ± 12.7*	58.5 ± 8.50*
CFU-GEMM	0.00 ± 0.00	3.50 ± 1.77	23.2 ± 3.38*	2.50 ± 0.50	0.00 ± 0.00	0.00 ± 0.00	16.4 ± 6.30*	17.4 ± 8.70*

(B)

	Control	MBL100	MBL200	MBL400	MBL800	MBL1600	FL	FL+MBL200
Total CFU	179 ± 3.89	232 ± 12.3	287 ± 7.89*	156 ± 31.1	128 ± 25.5	100 ± 20.0^{+}	280 ± 16.1*	309 ± 7.97*
CFU-GM	147 ± 16.5	150 ± 49.5	166 ± 35.5	117 ± 47.5	128 ± 45.0	66.5 ± 14.4	170 ± 12.7	177 ± 67.2
BFU/CFU-E	16.7 ± 6.42	66.0 ± 16.8	76.2 ± 16.8*	38.9 ± 6.20	0.00 ± 0.00^{+}	33.4 ± 13.2	45.7 ± 21.2	87.5 ± 17.5*
CFU-GEMM	0.00 ± 0.00	15.6 ± 5.00*	61.0 ± 11.8*	2.50 ± 0.51	0.00 ± 0.00	0.00 ± 0.00	64.0 ± 10.8*	44.7 ± 14.0*

Table 5.8 Effects of Mannose-Binding Lectin on the CFU Formation at Day 35

(A)

	Control	MBL100	MBL200	MBL400	MBL800	MBL1600	FL	FL+MBL200
Total CFU	136 ± 5.82	170 ± 8.76*	235 ± 11.9*	90.5 ± 1.56^{+}	94.5 ± 2.88^{+}	60.0 ± 7.07^{+}	214 ± 8.36*	247 ± 13.7*
CFU-GM	129 ± 28	155 ± 24.9	187 ± 17.2*	90.5 ± 18.5	60.0 ± 14.5	60.0 ± 12.0	172 ± 11.5*	196 ± 16.9*
BFU/CFU-E	6.30 ± 3.22	15.5 ± 5.49	30.4 ± 3.82*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	58.8 ± 5.71*	34.4 ± 4.42*
CFU-GEMM	0.00 ± 0.00	0.00 ± 0.00	14.6 ± 5.22*	3.5 ± 0.50	0.00 ± 0.00	0.00 ± 0.00	8.5 ± 3.00*	12.3 ± 2.82*

(B)

	Control	MBL100	MBL200	MBL400	MBL800	MBL1600	FL	FL+MBL200
Total CFU	123 ± 3.98	141 ± 15.0	257 ± 8.46*	134 ± 26.7	100 ± 20.0	66.0 ± 13.2	234 ± 40.4*	194 ± 7.38*
CFU-GM	123 ± 11.8	141 ± 42.0	257 ± 27*	134 ± 35.8	100 ± 26.0	66.0 ± 26.0^{+}	194 ± 29.5*	234 ± 54.2*
BFU/CFU-E	0.00 ± 0.00	16.0 ± 6.93	80.5 ± 20.1*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	35.4 ± 11.2*	66.5 ±14.9*
CFU-GEMM	0.00 ± 0.00	0.00 ± 0.00	41.7 ± 20.2*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	17.7 ± 5.42*	38.5 ± 18.1*

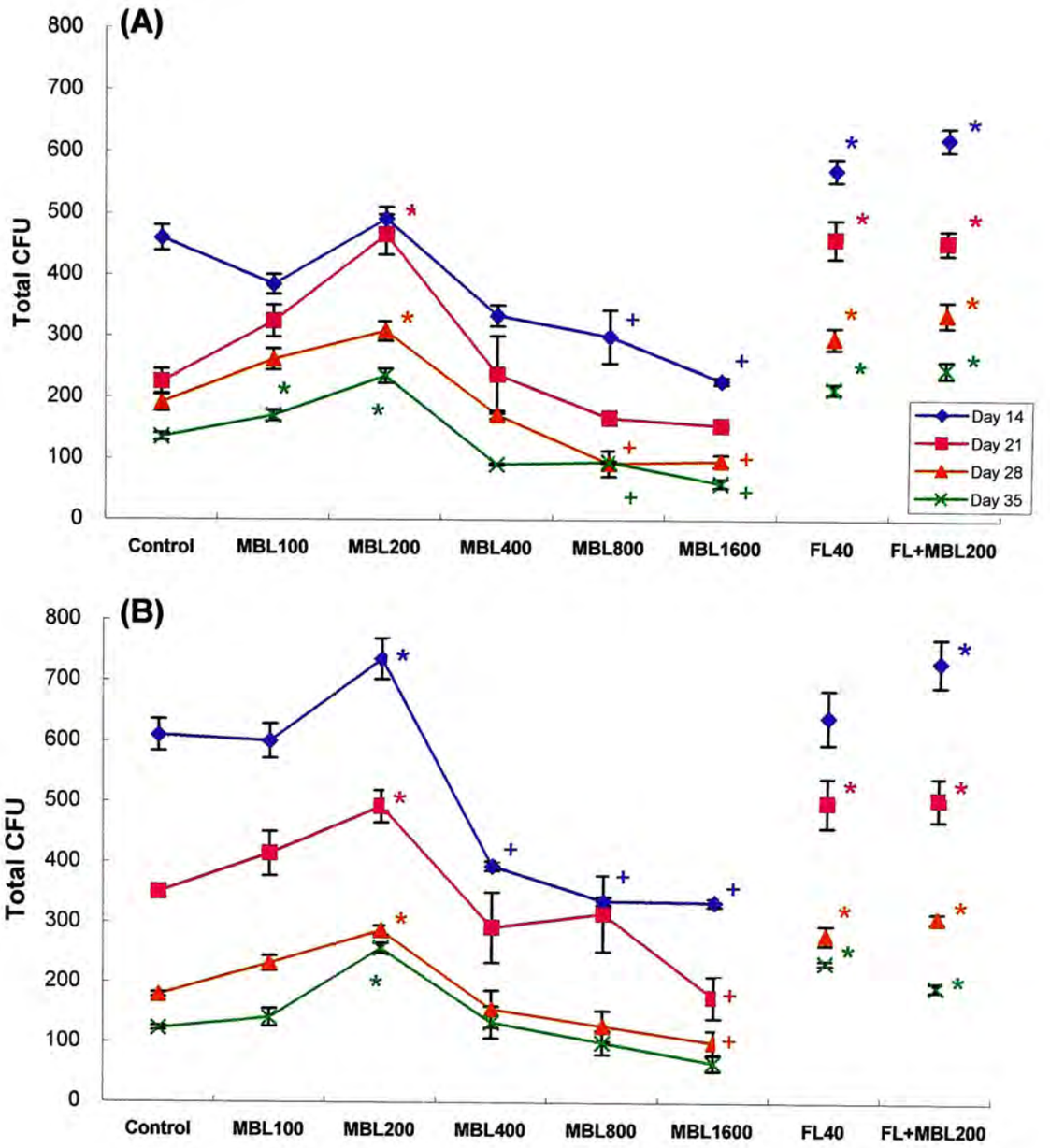


Figure 5.11 Effects of Mannose-Binding Lectin on CFU Formation

CFU were preserved in the presence of 200 ng/ml MBL or FL (* $p < 0.05$). However, the expansion of CFU decreased at higher concentrations of MBL ($^{+}$ $p < 0.05$). Results were presented as the mean number of CFU formed from 5 ml cultures at day 0 ± S.E. n = 8

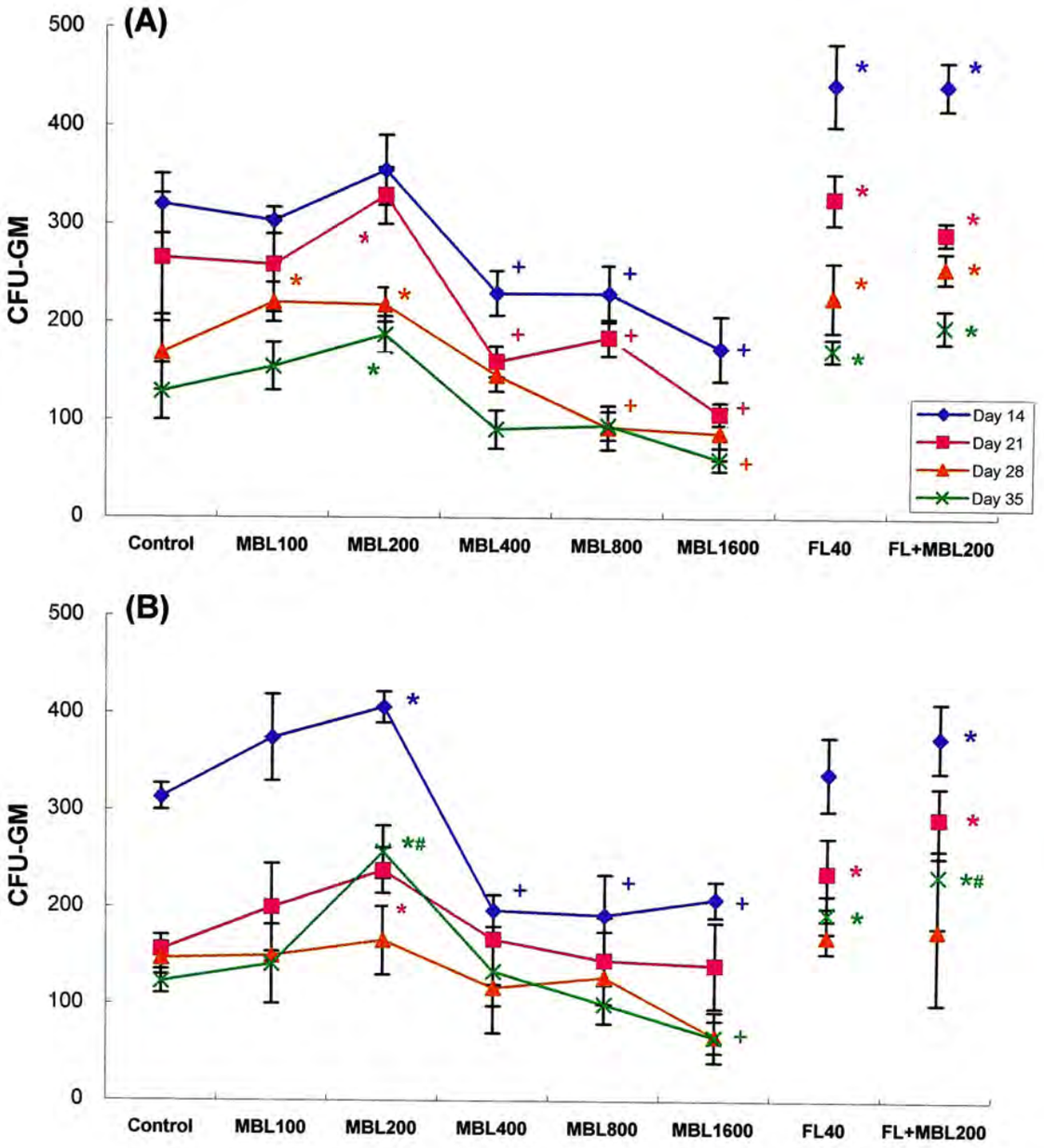


Figure 5.12 Effects of Mannose-Binding Lectin on CFU-GM

Significantly higher expansion of CFU-GM was seen in cultures with MBL200, FL and FL+MBL when compared to the control (* $p < 0.05$). CFU-GM formation in group A appeared to be suppressed by high concentrations (> 400 ng/ml) of MBL ($^{+}$ $p < 0.05$). At day 35, MBL200 and FL+MBL supported more efficient expansion of CFU-GM in $CD34^{+}$ cell cultures than FL ($^{\#}$ $p < 0.05$). n = 8

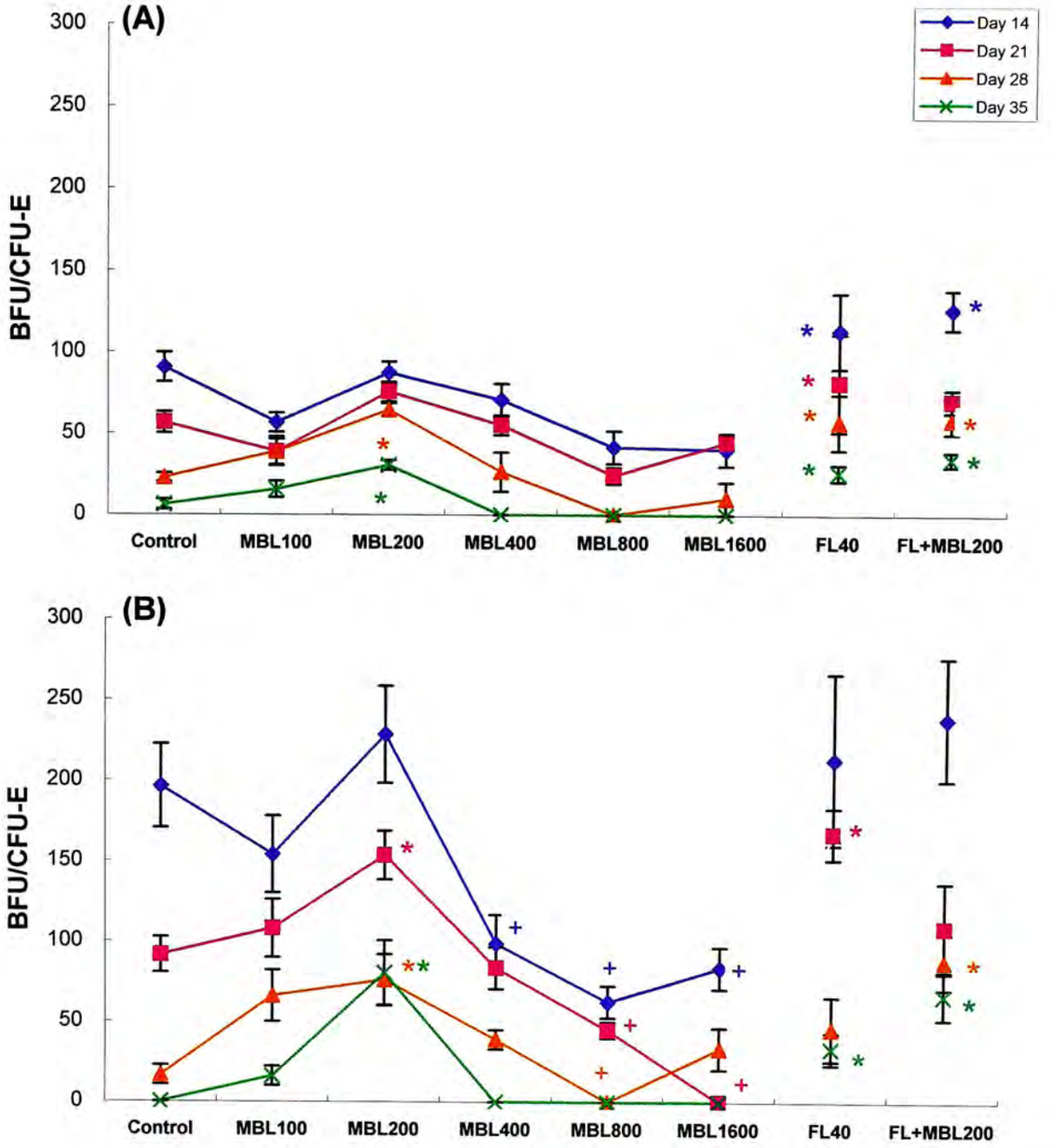


Figure 5. 13 Effects of Mannose-Binding Lectin on BFU/CFU-E

Significantly higher yields of BFU/CFU-E than the control were obtained in MBL200, FL40 and FL+MBL in both groups A and B (* $p < 0.05$). The yields of BFU/CFU-E decreased in MBL400, MBL800 and MBL1600 ($^{+}$ $p < 0.05$) in group B. n = 8

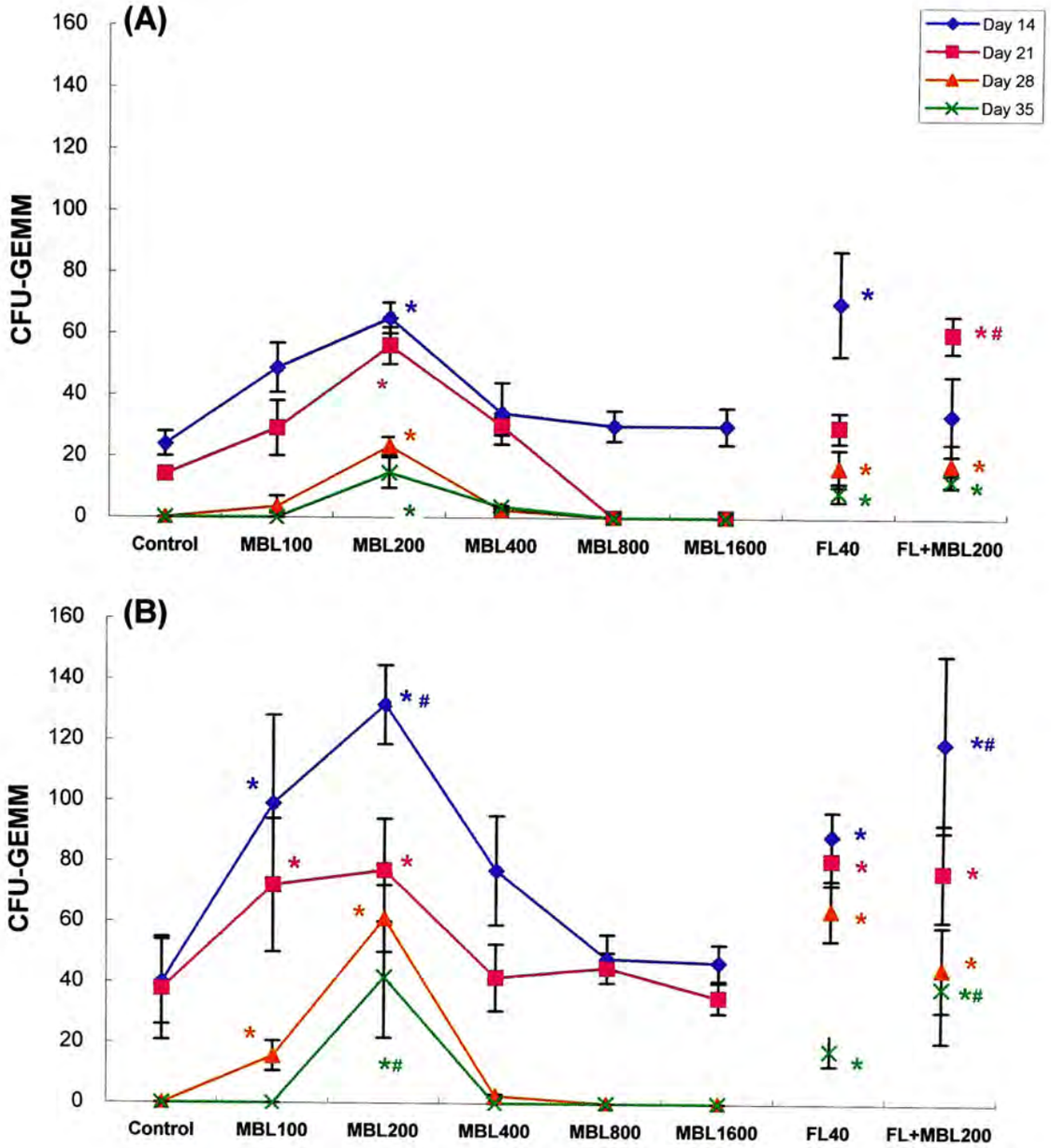


Figure 5. 14 Effects of Mannose-Binding Lectin on CFU-GEMM

MBL200, FL40 and FL+MBL supported higher yields of CFU-GEMM after 35 days of culture than the control in both (A) and (B) cultures and MBL100 also exerted this enhancing effect in (B) (* $p < 0.05$). The yields of CFU-GEMM in MBL200 were significantly higher than those in FL40 ($^{\#}$ $p < 0.05$) but had no differences with those in FL+MBL. n = 8

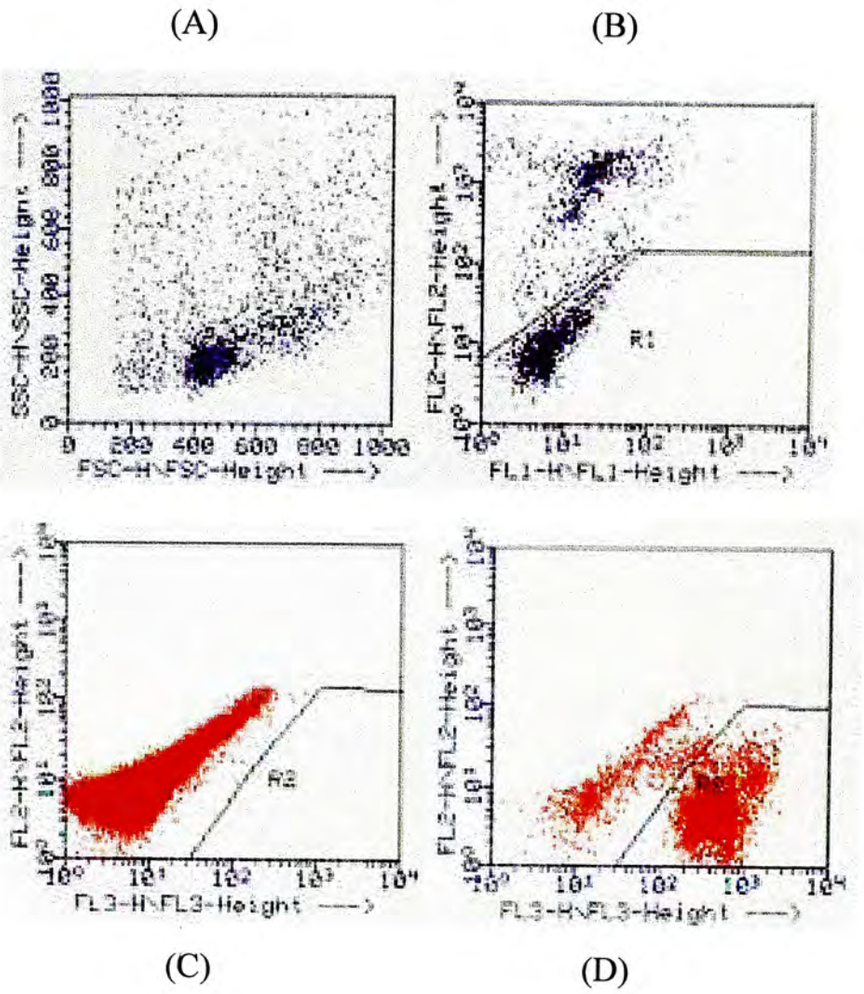


Figure 5.15 **Flow Cytometry Analysis of Bone Marrow Cells of Transplanted NOD/SCID Mice: Determination of Human CD45$^+$ Cells**

The percentage of human CD45$^+$ cells (FL-3) in the BM of transplanted mice was analyzed by flow cytometry. Dead cells, which were PI-positive were gated out (B). The expression of CD45 (cy5) (D) was determined by comparing with the isotypic control (C).

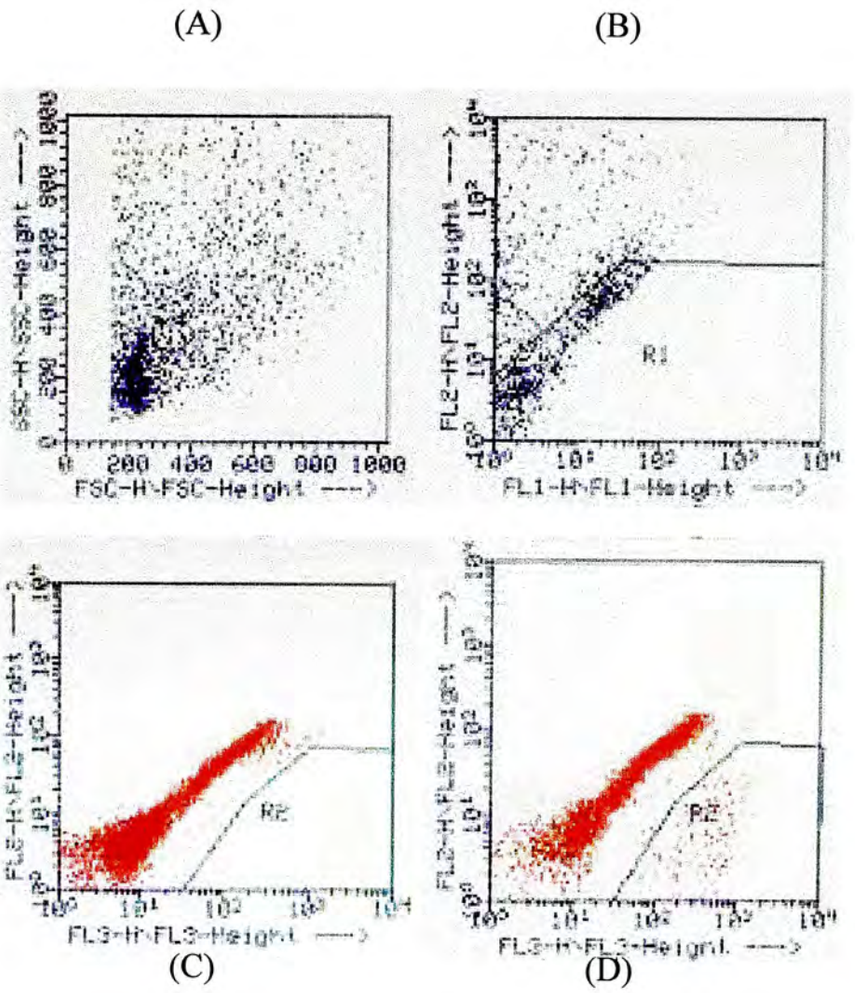
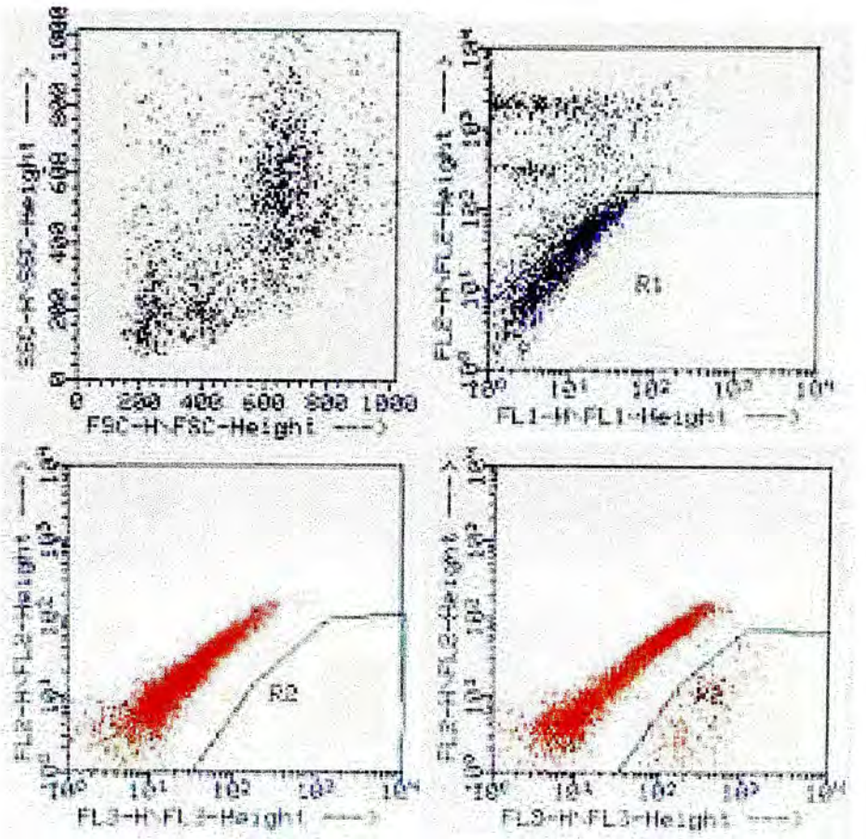


Figure 5.16 Flow Cytometry Analysis of Human CD45$^+$ Cells in the Spleen of Transplanted NOD/SCID Mice

The percentage of human CD45$^+$ cells in the spleen of transplanted mice was analyzed by flow cytometry. Dead cells, which were PI-positive were gated out (B). The expression of CD45 (cy5) (D) was determined by comparing with the isotypic control (C).

(A) (B)



(C) (D)

Figure 5.17 Flow Cytometry Analysis of Human CD45$^+$ Cells in Peripheral Blood of Transplanted NOD/SCID Mice

The percentage of human CD45$^+$ cells in the PB of transplanted mice was analyzed by flow cytometry. Dead cells, which were PI-positive were gated out (B). The expression of CD45 (cy5) (D) was determined by comparing with the isotypic control (C).

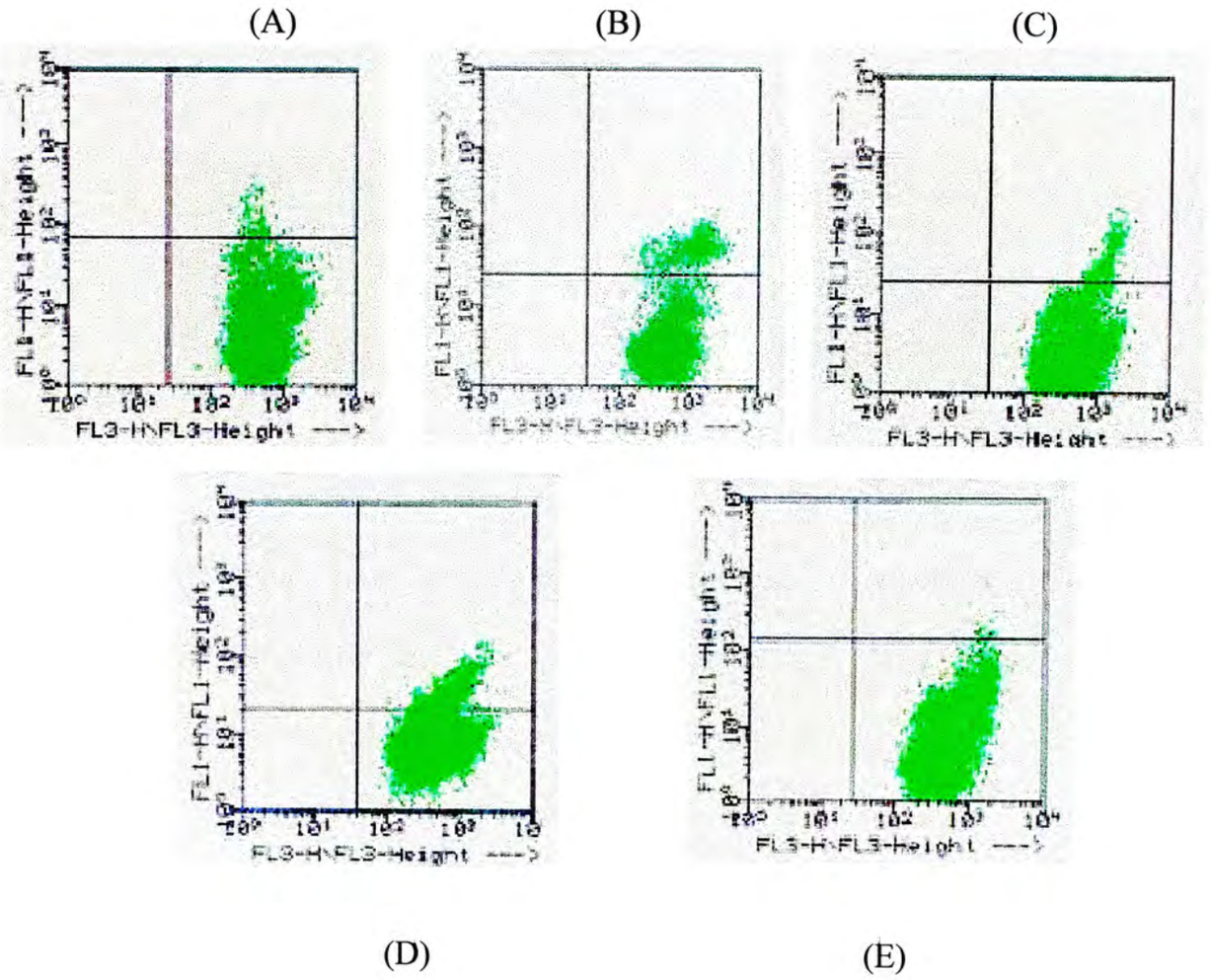


Figure 5.18 **Flow Cytometry Analysis of Bone Marrow Cells of Transplanted NOD/SCID Mice: Determination of Cell Subsets**

The expression (FL-1) of CD34 (A), CD33 (B), CD14 (C), CD19 (D) and CD61 (E) on human $CD45^+$ cells (FL-3) in BM of NOD/SCID mice engrafted with enriched CB $CD34^+$ cells cultured with TPO, SCF, FL in the presence of MBL for 14 days.

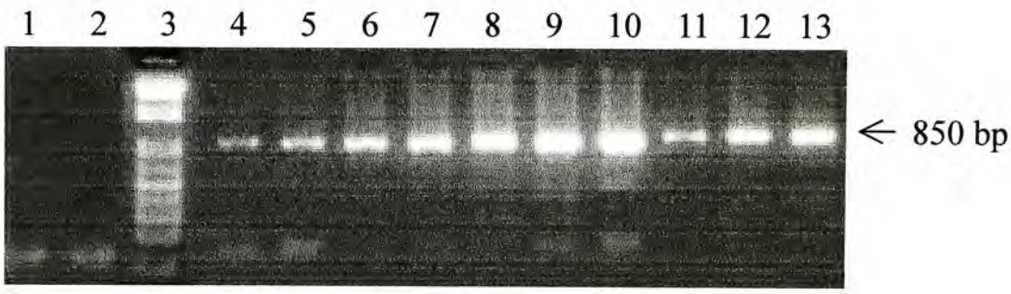


Figure 5.19 PCR Analysis of Human-Specific Alpha Satellite DNA in Chromosome 17 in Bone Marrow of NOD/SCID Mice Transplanted with Expanded Human Cells

Genomic DNA was extracted from the BM of transplanted mice and subjected to PCR. Gel electrophoresis illustrated that positive expression of human-specific DNA were observed in all mice. Human CB cells and non-transplanted NOD/SCID mouse BM cells were served as positive and negative controls, respectively. Different percentages of human cells were mixed with mouse cells and a set of standards was set up. 1 – H_2O, 2 – normal mouse BM cells, 3 – DNA size marker, 4 – 0.1% human DNA, 5 – 0.5% human DNA, 6 – 1.0% human DNA, 7 – 5.0% human DNA, 8 – 10% human DNA, 9 – 30% human DNA, 10 – 35% human DNA, 11 – 13: transplanted mouse BM cells (0.36%, 7.07%, 15.6% as measured by flow cytometry).

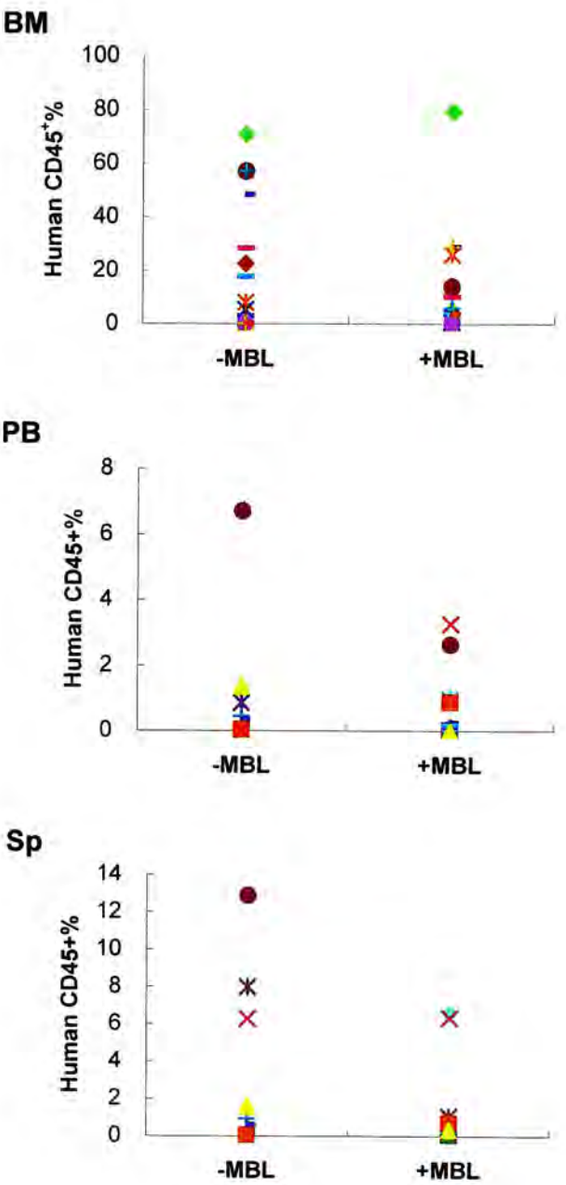


Figure 5.20 Engraftment of Expanded Human Cells in NOD/SCID Mice

Enriched $CD34^+$ cells expanded in the presence or absence of 200 ng/ml MBL were infused into irradiated NOD/SCID mice. After 6 weeks, the engraftment of human $CD45^+$ cells in the bone marrow (BM), peripheral blood (PB) and spleen (Sp) were quantified by flow cytometry. Results were expressed as the percentage of viable cells. n = 19 for each group.

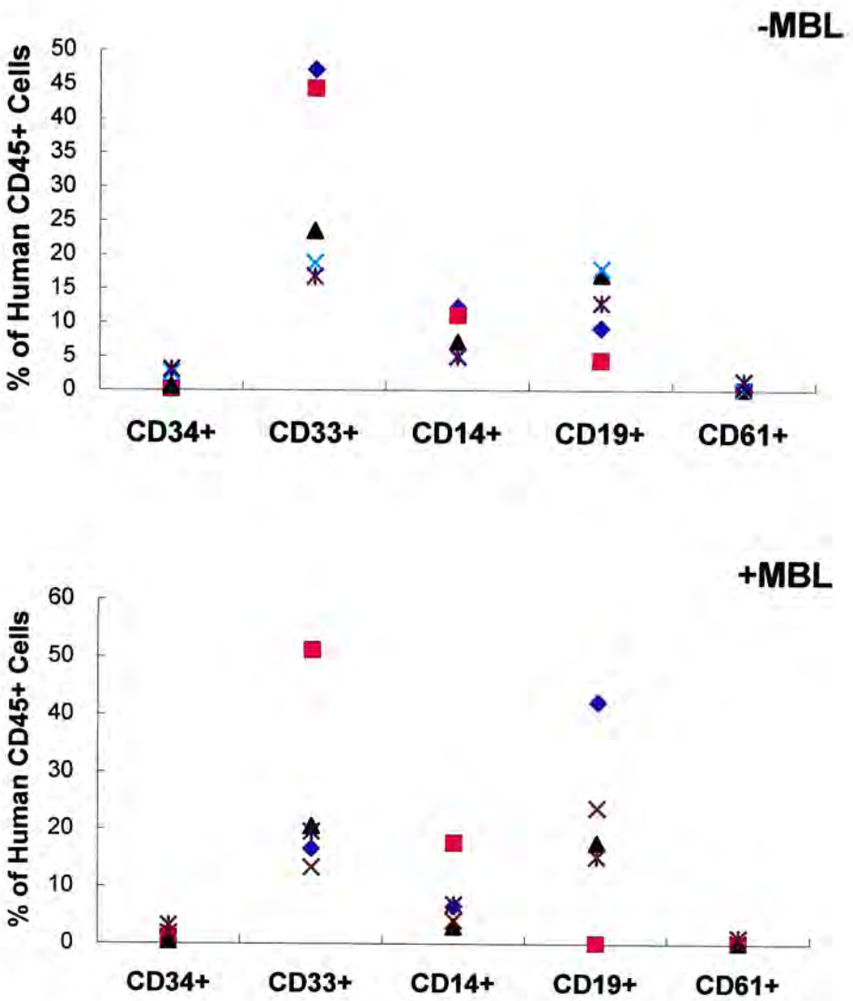


Figure 5.21 Subsets of Human Hematopoietic Cells in the Bone Marrow of Engrafted NOD/SCID Mice

The engraftment of human stem and progenitor ($CD34^+$), myeloid ($CD33^+$, $CD14^+$), lymphoid ($CD19^+$) and megakaryocytic ($CD61^+$) cells were detected in the BM of transplanted NOD/SCID mice after 6 weeks of transplantation. Results were expressed as the percentage of human $CD45^+$ cells. n = 5 for each group.

CHAPTER SIX

OPTIMIZATION OF CULTURE DURATION, CULTURE MEDIA, AUTOLOGOUS PLASMA AND CYTOKINE COMBINATIONS FOR THE PRECLINICAL *EX VIVO* EXPANSION OF HEMATOPOIETIC STEM AND PROGENITOR CELLS

Section 6.1 Results

The effects of culture duration, media, serum supplements and cytokine combinations on the *ex vivo* expansion of hematopoietic stem and progenitor cells were investigated. Enriched $CD34^+$ cells were cultured in four media, IMDM, X-Vivo 10, QBSF-60 and StemSpan, in the presence of four cytokine combinations consisting of TPO, SCF, FL, G-CSF and IL-6 with or without autologous CB plasma for 12 days. In separate experiments, 50 ng/ml TPO and SCF were replaced by 100 ng/ml in cultures with or without FL in QBSF-60. Selected groups of expanded cells were transplanted into NOD/SCID mice for the assessment of their engrafting ability.

6.1.1 Kinetics of Expansion

At day 0, the purity of enriched $CD34^+$ cells was 92.7 ± 1.30% (range 87.3 – 98.3%) and the viability of $CD34^+$ cells was 98.5 ± 0.53% (range 97.0 – 100%). At days 8 and 12, the viability of cultured cells as determined by trypan blue dye exclusion assay was 95.0 ± 0.96% (range 92.0 – 99.0%) and 92.7 ± 1.52 (range 82.5 – 99.0%), respectively. Total nucleated cells increased with duration of culture. The fold increases of total nucleated cells were significantly higher at day 12 of culture in all

treatments as compared to their respective cultures at day 8 ($p < 0.05$) (Figure 6.1).

Although the percentages of $CD34^+$ cells and $CD34^+CD38^+$ cells decreased with the duration of culture, a general increase in absolute numbers of these cells was seen at day 12 except in cultures with TSFG6 at which the yields of $CD34^+$ cells and $CD34^+CD38^-$ cells at day 12 appeared to be lower than those at day 8 (Table 6.1). The fold increases of $CD34^+$ cells in IMDM with TSF6; X-Vivo 10 with TSF and TSFG; QBSF-60 with TSF, TSFG and TSF6; and StemSpan with TSF and TSFG at day 12 were significantly higher than their respective cultures at day 8 while the fold increase of $CD34^+$ cells in IMDM and StemSpan with TSFG6 at day 12 was significantly lower than that at day 8 ($p < 0.05$) (Figure 6.2). In addition, the fold increases of $CD34^+CD38^-$ cells in cultures with TSF, TSFG and TSF6 at day 12 were higher than those at day 8 ($p < 0.05$ for IMDM with TSF, TSFG and TSF6; X-Vivo 10 with TSF and TSF6; QBSF-60 with TSF, TSFG and TSF6; and StemSpan with TSFG and TSF6). Similar to $CD34^+$ cells, the fold increases of $CD34^+CD38^-$ cells at day 12 were lower than those at day 8 in cultures with TSFG6 ($p < 0.05$ for X-Vivo 10 and QBSF-60) (Figure 6.3).

The colony-forming capacities of total CFU (CFU-GM + BFU/CFU-E + CFU-GEMM) and CFU-GM were higher at day 12 as compared to day 8 (Table 6.2). However, the trend on BFU/CFU-E was mixed, being higher at day 12 in some cultures and at day 8 in others. The multipotent CFU-GEMM were significantly higher at day 12 ($p < 0.05$) in all cultures except those in IMDM, X-Vivo 10 and QBSF-60 with cytokine treatment TSF; in all media with TSF6; and in X-Vivo 10 with TSFG6 where significant differences were not demonstrated.

$CD61^+CD41^+$ cells were significantly higher at day 12 comparing to day 8 results ($p < 0.05$), except in cultures with TSFG6 in four media at which only a trend was indicated (Figure 6.4). The numbers of CFU-MK were consistently higher in all cultures at day 12 when compared with those at day 8 ($p < 0.05$ for all media and treatments except in culture with TSFG6 in X-Vivo 10) (Figure 6.5 and 6.6). Considering the superior expansion of hematopoietic progenitors and subsets at day 12 when compared to those at day 8, we decided to present day 12 results in the following experiments to elucidate the effects of different media and growth factors on the *ex vivo* expansion of CB hematopoietic stem and progenitor cells (Section 6.1.2 – 6.1.7, Table 6.3 – 6.6, Figure 6.7 – 6.24).

6.1.2 Assessment of Culture Media

Four media, IMDM + 10% FCS, X-Vivo 10, QBSF-60 and StemSpan were studied in the presence of four cytokine combinations. The results were summarized in Table 6.3. After 12 days of culture, the expansion of total nucleated cells was generally more efficient in cultures with StemSpan serum-free medium. The fold increases of total nucleated cells were significantly higher in StemSpan when compared to IMDM ($p < 0.05$ for TSF, TSFG and TSF6), X-Vivo 10 ($p < 0.05$ for all treatments) and QBSF-60 ($p < 0.05$ for TSFG) (Figure 6.7).

Our results showed that QBSF-60 supported the highest expansion of both $CD34^+$ cells and $CD34^+CD38^-$ cells (Table 6.3). Significantly higher percentages of both $CD34^+$ and $CD34^+CD38^-$ cells were observed in QBSF-60 cultures when compared to those in IMDM, X-Vivo 10 and StemSpan ($p < 0.05$). QBSF-60 enhanced significantly higher yields and fold increases of these cells than other media ($p <$

0.05), except StemSpan with cytokine treatments TSF and TSFG, at which a trend was shown (Table 6.3 and Figure 6.8).

The total colony-forming capacity appeared to be superior in QBSF-60 and StemSpan cultures when compared to IMDM (vs QBSF-60, $p < 0.05$ for cytokine treatments TSF, TSFG and TSF6; vs StemSpan, $p < 0.05$ for TSFG) and X-Vivo 10 ($p < 0.05$ for all treatments) (Figure 6.9). The fold increases of CFU-GM in QBSF-60 were higher than those in other three media ($p < 0.05$ for X-Vivo 10 with TSF, TSFG and TSF6; and StemSpan with TSF6) while the fold increase of CFU-GM in culture in StemSpan with TSF6 was significantly higher than that in X-Vivo10 ($p < 0.05$) (Figure 6.9). QBSF-60 also supported higher fold increases of BFU/CFU-E when compared to IMDM ($p < 0.05$ for TSF6), X-Vivo 10 ($p < 0.05$ TSF, TSF6 and TSFG6) and StemSpan ($p < 0.05$ for TSF6) while StemSpan produced higher fold increase of BFU/CFU-E than IMDM with cytokine treatment TSF ($p < 0.05$) (Figure 6.9). Furthermore, the fold increases of multipotent CFU-GEMM were higher in QBSF-60 and StemSpan, particularly in the former medium (Figure 6.9). The fold increases of CFU-GEMM in StemSpan cultures were significantly higher than IMDM ($p < 0.05$ for TSF6) and X-Vivo 10 ($p < 0.05$ for TSFG, TSF6 and TSFG6). QBSF-60 produced the highest yield of CFU-GEMM when compared to cultures in IMDM ($p < 0.05$ for all treatments), X-Vivo 10 ($p < 0.05$ for all treatments) and StemSpan ($p < 0.05$ for TSFG, TSF6 and TSFG6) (Table 6.3). The yields of CFU-GEMM in QBSF-60 were 119 – 437%, 196 – 2386% and 63 – 176% of those in IMDM, X-Vivo 10 and StemSpan, respectively.

Considering the megakaryocytic lineage, the expansion appeared to be the most

effective in X-Vivo 10 (Table 6.3 and Figure 6.10). X-Vivo 10 produced the highest percentage of $CD61^{+}41^{+}$ cells in all cytokine treatments (Figure 6.10), and higher yield of these cells when compared to IMDM ($p < 0.05$ for all treatments), QBSF-60 ($p < 0.05$ for TSFG, TSF6 and TSFG6) and StemSpan ($p < 0.05$ for TSFG, TSF6 and TSFG6) (Table 6.3). Similar trends were observed in the expansion of CFU-MK. The fold increases of CFU-MK in X-Vivo 10 cultures were significantly higher than those in IMDM ($p < 0.05$ for all treatments), QBSF-60 ($p < 0.05$ for TSFG, TSF6 and TSFG6) and StemSpan ($p < 0.05$ for all treatments) (Figure 6.10). The yields of CFU-MK in X-Vivo 10 with different cytokine treatments were 125 – 198% of those in the other three media (Table 6.3).

6.1.3 Effects of Autologous Cord Blood Plasma

Enriched $CD34^{+}$ cells were cultured in IMDM containing either 10% FCS or 10% autologous CB plasma, or in X-Vivo 10, QBSF-60 and StemSpan with or without autologous plasma in the presence of four cytokine treatments. Significant increases in total nucleated cells were observed in cultures containing autologous plasma in X-Vivo 10, QBSF-60 and StemSpan ($p < 0.05$ for all treatments except in QBSF-60 and StemSpan with cytokine treatment TSFG) (Table 6.4). In contrast, no significant difference was observed in IMDM containing FCS or autologous plasma except in TSF where a significant increase was demonstrated in the presence of autologous plasma.

Our results demonstrated that no significant differences were observed in the expansion of $CD34^{+}$ cells when FCS was replaced by autologous plasma in IMDM cultures (Figure 6.11). However, the addition of autologous plasma to X-Vivo 10 (p

< 0.05 for all treatments), QBSF-60 ($p < 0.05$ for TSF, TSFG and TSFG6) and StemSpan ($p < 0.05$ for all treatments) serum-free media decreased both the percentages and the yields of $CD34^+$ cells substantially (Figure 6.11). The fold increases of $CD34^+$ cells in cultures with autologous plasma were 41.4 – 75.8%, 23.7 – 43.1% and 35.9 – 47.5% of those without plasma in X-Vivo 10, QBSF-60 and StemSpan, respectively. Similar to $CD34^+$ cells, the expansion efficiency of $CD34^+CD38^-$ cells were not different in the presence of FCS or autologous plasma in IMDM cultures (Figure 6.12). The percentages and the yields of $CD34^+CD38^-$ cells decreased drastically when autologous plasma was added to cultures in the three serum-free media ($p < 0.05$) (Figure 6.12). The fold increases of these cells in X-Vivo 10, QBSF-60 and StemSpan with autologous plasma were only 2.02 – 20.6%, 12.7 – 20.4% and 3.37 – 11.0% of those in their respective cultures without autologous plasma.

No significant difference was found in the expansion of total CFU and specific lineages of CFU in the presence of FCS or autologous plasma in IMDM (Table 6.5). In serum-free media, there was also no significant difference in the expansion of total CFU in the presence or absence of autologous plasma (Table 6.5). The addition of autologous plasma to the three serum-free media enhanced the expansion of CFU-GM and BFU/CFU-E. The expansion of CFU-GM and BFU/CFU-E were significantly more efficient in the presence of autologous plasma in X-Vivo 10 ($p < 0.05$ for all treatments), QBSF-60 ($p < 0.05$ for all treatments) and StemSpan ($p < 0.05$ for TSF and TSFG6) (Table 6.5). On the other hand, the fold increases of CFU-GEMM dropped in a dramatic manner when autologous plasma was added to cultures in X-Vivo 10 ($p < 0.05$ for all treatments), QBSF-60 ($p < 0.05$ for all

treatments) and StemSpan ($p < 0.05$ for TSF, TSFG and TSFG6) (Table 6.5). The yields of CFU-GEMM in cultures with autologous plasma were 59.2 – 87.8%, 44.9 – 78.3% and 48.3 – 59.8% of those without plasma in X-Vivo 10, QBSF-60 and StemSpan, respectively.

The yields of megakaryocytic $CD61^+CD41^+$ cells were reduced significantly by the addition of autologous plasma in X-Vivo 10, QBSF-60 and StemSpan ($p < 0.05$ for all treatments except StemSpan with TSFG) (Figure 6.13). No significant differences were demonstrated in cultures in IMDM with FCS or autologous plasma (Figure 6.13). The fold increases of CFU-MK were also decreased when autologous plasma was added to the three serum-free media ($p < 0.05$ for all treatments except StemSpan with TSFG) (Figure 6.14). The yields of CFU-MK in cultures with autologous plasma were 85.0 – 96.0%, 84.9 – 96.0% and 80.9 – 92.7% of those without plasma in X-Vivo 10, QBSF-60 and StemSpan, respectively.

6.1.4 Effects of Granulocyte-Colony Stimulating Factor

In order to study the effects of G-CSF on the expansion of CB $CD34^+$ cells, enriched $CD34^+$ cells were cultured in four media, IMDM + 10% FCS, X-Vivo 10, QBSF-60 and StemSpan, in the presence of TSF or TSF6 with or without G-CSF (Table 6.3). In all media, G-CSF increased total nucleated cells to 136 – 273% ($p < 0.01$) (Figure 6.15) but decreased the percentage of $CD34^+$ cells to 44.0 – 71.0% ($p < 0.05$) after 12 days of culture (Table 6.3). As a result, there was no significant difference in the absolute number of $CD34^+$ in the presence or absence of G-CSF except cultures in X-Vivo 10 with TSF, at which G-CSF significantly increased the yield of $CD34^+$ cells ($p < 0.01$) (Figure 6.16). Moreover, the percentages of $CD34^+CD38^-$ cells in

cultures with TSFG and TSFG6 dropped significantly to 43.0 – 60.6% of those in cultures with TSF ($p < 0.05$ for X-Vivo 10, QBSF-60 and StemSpan) and 39.6 – 68.4% of those with TSF6 ($p < 0.05$ for IMDM, X-Vivo 10 and QBSF-60), respectively (Table 6.3). The significant decreases in the fold increases of $CD34^+CD38^-$ cells were also observed in X-Vivo 10 and StemSpan with TSF6 ($p < 0.05$) (Figure 6.16).

G-CSF appeared to enhance the expansion of CFU in all cultures (Table 6.3 and Figure 6.17). In cultures with TSF, total CFU, CFU-GM, BFU/CFU-E and CFU-GEMM were significantly elevated when G-CSF was added ($p < 0.05$). Similar results were obtained in cultures with TSF6, but significant increases were only observed in total CFU in cultures in StemSpan; CFU-GM in IMDM and QBSF-60; and CFU-GEMM in IMDM, QBSF-60 and StemSpan ($p < 0.05$).

Our results demonstrated that the addition of G-CSF to cultures with TSF did not change the yields of $CD61^+CD41^+$ cells in all media (Table 6.3), although the percentages of these cells declined ($p < 0.05$ for IMDM, QBSF-60 and StemSpan) (Figure 6.18). The yields of $CD61^+CD41^+$ cells in cultures with TSFG6 decreased to 42.5 – 70.7% of those with TSF6 (Table 6.3). However, G-CSF enhanced the formation of CFU-MK to 139 – 218% ($p < 0.05$ for all treatments except X-Vivo 10 with TSF6) (Table 6.3 and Figure 6.18).

6.1.5 Effects of Interleukin-6

Effects of IL-6 were studied in the four media with either TSF or TSFG (Table 6.3). IL-6 appeared to enhance the expansion of total nucleated cells. Significant increases

were observed in cultures with TSF in all media ($p < 0.05$), a trend was demonstrated in cultures with TSFG or TSFG6 (Figure 6.15). The presence of IL-6 increased the percentages ($p < 0.05$ for X-Vivo 10 with TSFG) and the yields ($p < 0.01$ for IMDM with TSF, QBSF-60 with TSF and TSFG) of $CD34^+$ cells (Table 6.3 and Figure 6.16). IL-6 affected the yields of $CD34^+CD38^-$ cells in a similar fashion ($p < 0.05$ for QBSF-60 with TSF).

The yields of total CFU were higher in all cultures with TSF6 when compared to ones with TSF ($p < 0.05$) (Table 6.3). The fold increases of total CFU in cultures with TSF6 were 150 – 183% of those in cultures with TSF (Figure 6.17). However, there was no significant difference in between TSFG and TSFG6 in the yields of CFU. The addition of IL-6 enhanced the expansion of CFU-GM ($p < 0.05$ for IMDM with TSF and QBSF-60 with TSFG), BFU/CFU-E ($p < 0.05$ for IMDM with TSF, X-Vivo 10 with TSFG and QBSF-60 with TSF) and CFU-GEMM ($p < 0.05$ for IMDM and QBSF-60 with TSF).

IL-6 did not consistently increase the expansion of $CD61^+CD41^+$ cells. Significant increases were only observed in IMDM and QBSF-60 with TSF ($p < 0.05$) (Figure 6.18). IL-6 enhanced the formation of CFU-MK in all media. Significant increases were observed in cultures with TSFG ($p < 0.05$) (Figure 6.18). The yields of CFU-MK in cultures with IL-6 were 132 – 169% as compared to cultures without IL-6.

6.1.6 Effects of Increased Dosage of Thrombopoietin and Stem Cell Factor

In the attempt to optimize the working concentration of TPO and SCF in the specific serum-free medium, the effects of two dosage (50 ng/ml and 100 ng/ml) of TPO and

SCF on the expansion of eight independent CB samples were studied in cultures with QBSF-60 in the presence of TPO, SCF, G-CSF, with or without FL. After dosage increment of TPO and SCF, the fold increase of total nucleated cells were elevated, together with the increases in the percentage of $CD34^+$ cells ($p < 0.01$ for cytokine treatment TSFG). The yields and fold increases of $CD34^+$ cells were also elevated in both cytokine treatments TSG and TSFG. Similar results for $CD34^+CD38^-$ cells were observed (Table 6.6 and Figure 6.19).

When 50 ng/ml TPO and SCF were replaced by 100 ng/ml TPO and SCF, all CFU formation including CFU-GM, BFU/CFU-E and CFU-GEMM elevated significantly in TSG ($p < 0.05$). In TSFG, significantly higher expansion was only observed in BFU/CFU-E ($p < 0.05$) (Table 6.6 and Figure 6.20).

For the megakaryocytic lineage, the percentage and yield of $CD61^+CD41^+$ cells in cultures with 100 ng/ml TPO and SCF were higher than those with 50 ng/ml TPO and SCF ($p < 0.01$ for TSG) (Table 6.6). Moreover, the expansion of CFU-MK was higher in cultures with 100 ng/ml TPO and SCF when compared to cultures with 50 ng/ml TPO and SCF ($p < 0.05$ for TSFG) (Table 6.6 and Figure 6.21).

6.1.7 Effects of Flt-3 Ligand

Enriched $CD34^+$ cells from eight CB samples were cultured in QBSF-60 in the presence of 50 ng/ml or 100 ng/ml TPO and SCF, 40 ng/ml G-CSF, with or without 80 ng/ml FL to investigate the effects of FL on the *ex vivo* expansion of CB $CD34^+$ cells. The addition of FL to cultures containing both dosages of TPO and SCF, TSG and TSG100, significantly enhanced the expansion of total nucleated cells ($p < 0.01$)

(Table 6.6 and Figure 6.19). The fold increases of total nucleated cells in cultures with TSFG and TSFG100 were up to 173% of those in cultures with TSG and TSG100.

Both the percentage and the yield of $CD34^+$ cells elevated with the addition of FL to the cultures ($p < 0.01$) (Table 6.6 and Figure 6.19). The fold increase of $CD34^+$ cells were increased to 392% and 361% in cultures with TSFG and TSFG100, respectively. Adding FL also led to significant increases of the percentage and the yield of early $CD34^+CD38^-$ cells ($p < 0.05$) (Table 6.6 and Figure 6.19). The fold increases of $CD34^+CD38^-$ cells were increased to 264 – 273% when cultures were supplemented with FL.

FL enhanced the expansion of all lineages of CFU (Table 6.6 and Figure 6.20). Significant increases of total CFU, CFU-GM, BFU/CFU-E and CFU-GEMM were illustrated in all cultures with FL ($p < 0.01$).

However, no significant difference was observed in the yields of $CD61^+CD41^+$ cells in all cultures with or without FL although the percentages of these cells were significantly higher in TSFG and TSFG100 when compared to TSG and TSFG, respectively (Table 6.6). In contrast, the expansion of CFU-MK was better when FL was added to cultures ($p < 0.01$) (Table 6.6 and Figure 6.21).

6.1.8 Transplantation of Expanded Cells into NOD/SCID Mice

The engraftment capacity of expanded cells in the NOD/SCID mouse model was investigated. Cryopreserved CB $CD34^+$ cells were cultured in QBSF-60 medium

with TSG or TSFG for 12 days. Expanded cells from five CB cultures with each cytokine treatment were transplanted into 16 mice. In the TSG group, 13 mice remained alive after 6 weeks of transplantation and 11 of them showed engraftment of human cells. Flow cytometry and PCR were performed for the analysis. The percentages of human $CD45^+$ cells in the BM, spleen and PB of these mice as measured by flow cytometry were 8.13 ± 4.80% (range 0.01 – 52.6%), 1.77 ± 0.21 (range 1.47 – 2.17%) and 1.87 ± 1.29% (range 0.54 – 4.45%), respectively (Figure 6.22). In PCR analysis, all of these mice showed positive results, indicating that their BM contained cells with human-specific alpha-satellite DNA. The subsets of human hematopoietic cells were also detected in the BM of transplanted mice which contained more than 1% human $CD45^+$ cells. The human $CD45^+$ cells were consisted of 9.64 ± 2.84% (range 4.82 – 14.6%) $CD34^+$ cells, 21.9 ± 0.95% (range 20.3 – 23.6%) $CD33^+$ cells, 11.2 ± 1.58% (range 8.97 – 14.2%) $CD14^+$ cells, 24.9 ± 8.02% (range 9.64 – 36.8%) $CD19^+$ cells and 0.71 ± 0.35% (range 0.02 – 1.13%) $CD61^+$ cells (Figure 6.23).

In the TSFG group, 12 mice remained alive for 6 weeks after transplantation and engraftment was observed in all of them. The percentages of human $CD45^+$ cells in the BM, spleen and PB of these mice as measured by flow cytometry were 11.0 ± 3.95% (range 0.19 – 35.9%), 7.57 ± 1.26 (range 5.25 – 9.57%) and 1.59 ± 0.34% (range 1.10 – 2.25%), respectively (Figure 6.22). All of these mice demonstrated positive results in PCR analysis. In the BM of these mice, the subpopulations of human $CD45^+$ cells were consisted of 9.63 ± 4.30% (range 4.10 – 18.1%) $CD34^+$ cells, 22.8 ± 6.25% (range 10.7 – 31.5%) $CD33^+$ cells, 8.87 ± 2.48% (range 4.18 – 12.6%) $CD14^+$ cells, 28.7 ± 15.9% (range 12.2 – 60.5%) $CD19^+$ cells and 0.66 ±

0.12% (range 0.51 – 0.89%) $CD61^+$ cells (Figure 6.23). Although the mean levels of human $CD45^+$ cell percentage engrafted in the BM of mice transplanted with TSFG-expanded cells were higher than those with TSG-expanded cells, the percentages of $CD45^+$ cells as well as the subsets were not statistically different by performing Wilcoxon Sign Rank test comparison ($p = 0.0757$). On the other hand, the percentage of human $CD45^+$ cells in the spleen of mice transplanted with TSFG-expanded cells was significantly higher than that with TSG-expanded cells ($p = 0.0161$).

Section 6.2 Discussion

This study on the optimization of culture conditions for the expansion was conducted to establish a clinically applicable protocol for the ex *vivo* expansion of CB hematopoietic stem and progenitor cells. The expansion of CB $CD34^+$ cells to different lineages of hematopoietic cells was systematically assessed with various parameters such as culture duration, serum-free media, autologous supplements and cytokines.

The quality and quantity of cell products from *ex vivo* expansion cultures largely depends on the balance between the proliferative and maturation activities of seeded $CD34^+$ cells, which are affected by the duration of culture. A prolonged duration of culture increases cost, the risk of contamination and the exhaustion of early multipotent progenitors. Most clinical expansions were performed within the period of seven to twelve days (Bertolini *et al.* 1997a, Kögler *et al.* 1999, Paquette *et al.* 2000, Stiff *et al.* 2000). In this study, the yields of most progenitor cell populations, in particular those of megakaryocytic progenitors, were shown to be higher at day 12 when compared to those at day 8. More significantly, the early progenitors as

identified by the $CD34^+CD38^-$ cells and CFU-GEMM at day 12 were either higher or similar to their respective cultures at day 8. Möbest *et al.* (1999) and Bhatia *et al.* (1997b) reported that transplantation of cell products from expansion of over four and nine days resulted in the failure of stem cell engraftment in NOD/SCID mice, respectively. However, using the optimized media/cytokines and culture duration of 12 days, we obtained stable engraftments and human cell hematopoietic reconstitution in the NOD/SCID mouse transplantation model.

Traditionally, FCS was used in most expansion protocols. However, in order to avoid the risk of allergic reagents, viral contamination, variation between batches and regulatory issues of FCS (Sandstrom *et al.* 1996, Kögler *et al.* 1998), serum-deprived medium would be preferably used in clinical expansion. A number of serum-free media have been developed over the last few years. Several studies demonstrated that serum-free medium could support better growth for hematopoietic cells (Sandstrom *et al.* 1996, Kögler *et al.* 1998, Lefebvre *et al.* 1999, Möbest *et al.* 1999, Qiu *et al.* 1999, van den Oudenrijn *et al.* 2001). Three commercially available serum-free media were assessed in this study, in comparison with serum-containing IMDM. Autologous plasma has been used in some clinical expansion as an alternative nutrient supplement for replacing FCS (Brugger *et al.* 1995, Alcorn *et al.* 1996, Bertolini *et al.* 1997b, Kögler *et al.* 1999). If proven to be suitable, CB plasma can be obtained during the initial processing procedure of volume reduction and banked together with the CB unit.

Our results demonstrated that IMDM with 10% FCS was not superior to other serum-free media on the expansion of CB $CD34^+$ cells. In the serum free media, the

addition of CB plasma enhanced the expansion of more mature progenitors, giving higher yields of CFU-GM and BFU/CFU-E at the expense of primitive $CD34^+CD38^-$ cells and CFU-GEMM. Our results are in agreement with previous studies that CB plasma promoted differentiation of stem cells into myeloid and erythroid cells (Bertolini *et al.* 1994, Ruggieri *et al.* 1994). These data suggested that some factors in CB plasma might promote maturation and differentiation of hematopoietic cells and hence, reduce the expansion of multipotent CFU-GEMM.

It is of great importance to obtain a serum-free medium which promotes the large-scale *ex vivo* expansion for clinical use. In this study, QBSF-60 and to a lesser degree StemSpan were shown to support high yields of early progenitors as well as committed myeloid and erythroid cells. Qiu *et al.* (1999) showed that QBSF-60 with a simple cytokine combination containing TPO, SCF and FL supported simultaneous expansion of various CB hematopoietic cell compartments. Almeida-Porada *et al.* (2000) also demonstrated hematopoietic engraftment in the human-to-sheep xenograft model after expansion in QBSF-60. Taken together, the data confirmed that QBSF-60 might be a good choice of serum-free medium for the expansion of CB $CD34^+$ cells in a clinical setting.

Interestingly, the expansion of megakaryocytic progenitors was consistently higher in X-Vivo 10. X-Vivo 10 has been used clinically in the expansion of CB (Kögler *et al.* 1999) and mobilized PB hematopoietic stem and progenitor cells (Williams *et al.* 1996, Bertolini *et al.* 1997b). van den Oudenrijn *et al.* (2001) reported that StemSpan supported better expansion of $CD41^+$ cells than IMDM with 10% human AB plasma or CellGro SCGM which was another serum-free medium. These reports reinforced

the observation that different medium might support the expansion of different lineages of hematopoietic cells. In our experimental conditions, X-Vivo 10 appeared to be the most suitable medium for the expansion of megakaryocytic progenitors with $CD61^+CD41^+$ cells and CFU-MK expansion up to 2.79×10^6 cells per 2×10^4 seeded $CD34^+$ cells and 684-fold, respectively.

The combination of three early-acting cytokines, TPO, SCF and FL, has been used in many culture systems using various culture media for both pre-clinical (Briddell *et al.* 1997, Luens *et al.* 1998, Williams *et al.* 1998, Möbest *et al.* 1999, Murray *et al.* 1999, Kobari *et al.* 2000, Kratz-Albers *et al.* 2000, Lefebvre *et al.* 2000, Lewis *et al.* 2000, Shih *et al.* 2000, Xu *et al.* 2001) and clinical (Bertolini *et al.* 1997b, Kögler *et al.* 1999, McNiece *et al.* 2000b, Paquette *et al.* 2000, Stiff *et al.* 2000) studies with or without other cytokines. This combination was shown to have an enhancing effect on the survival and proliferation of early stem cells (Petzer *et al.* 1996b, Kobari *et al.* 1998, Luens *et al.* 1998), suppressing apoptosis of $CD34^+$ cells and recruiting primitive cells into rapid division (Murray *et al.* 1999). The combination of these cytokines with either G-CSF or IL-6 or both was further evaluated in this study. The addition of G-CSF appeared to enhance the expansion of some cell compartments but inhibit others. G-CSF increased the expansion of total nucleated cells and likely enhanced the maturation to some extent. The effects of this cytokine were mostly favorable in QBSF-60 and X-Vivo 10. Lefebvre *et al.* (2000) confirmed that G-CSF did not improve MK production, but rather favored the myeloid lineages. Kobari *et al.* (2000) cultured CB $CD34^+$ cells in serum-free IMDM supplemented with the same cytokine combination, TSFG with slightly different cytokine concentrations (100 ng/ml TPO, 100 ng/ml SCF, 100 ng/ml FL and 10 ng/ml G-CSF) expanded total

nucleated cells, $CD34^+$ cells, CFU-GM and BFU-E after 14 days of culture to 1,613-, 119-, 278- and 50-fold, respectively. In our conditions TSFG, expansions of total nucleated cells, $CD34^+$ cells, CFU-GM and BFU-E were 1,680-, 196-, 741- and 478-fold, respectively after 12 days of culture, again showing that serum-free medium might be superior to traditionally used IMDM. They also found that expanded cells were able to engraft in NOD/SCID mice and differentiate into myeloid, erythroid and lymphoid cells. Considering that G-CSF has been applied extensively for clinical use (Stahel *et al.* 1994, Klumpp *et al.* 1995, Lane *et al.* 1995, Li *et al.* 1999), its inclusion in the cytokine cocktail should be considered.

The addition of IL-6 produced favorable expansion outcomes including SCID-repopulating cells as reported in other studies (Sui *et al.* 1999, Kusadasi *et al.* 2000, Ueda *et al.* 2000). Kusadasi *et al.* (2000) compared different combinations containing the four cytokines, TPO, SCF, FL and IL-6. The expansion of both early and late progenitors was most efficient using all the four cytokines. Expanded cells were shown to be able to engraft in NOD/SCID mice (Kusadasi *et al.* 2000, Ueda *et al.* 2000). Ueda *et al.* (2000) reported that a substantial increase of human $CD45^+$ cells engrafted in NOD/SCID mice was observed when IL-6 was added to the cultures with TPO, SCF and FL. Up until now, IL-6 has not been widely used for patient treatments (Schrezenmeier *et al.* 1995, Bracho *et al.* 2001) or clinical *ex vivo* expansion (Bertolini *et al.* 1997b). Further evaluation and development IL-6 for the expansion of hematopoietic stem and progenitor cells might be desirable.

Recent studies have assessed the use of high dosage (100 ng/ml) of TPO and SCF for the *ex vivo* expansion of stem and progenitor cells for clinical applications (McNiece

et al. 1999, Reiffers *et al.* 1999, Paquette *et al.* 2000, McNiece *et al.* 2001). The effects of two dosage (50 ng/ml and 100 ng/ml) of TPO and SCF were investigated in the presence of QBSF-60 serum-free medium. The high dose TPO and SCF increased the expansion of all cell compartments without the exhaustion of early progenitors, showing that high dosage of TPO and SCF might be applied in the clinical expansion protocols.

In Chapter Four, we demonstrated that FL supported the expansion of both early and megakaryocytic progenitors in serum-containing IMDM. In this chapter, we further investigated the effects of FL in the serum-free QBSF-60 medium. The expansion of total nucleated cells, $CD34^+$ cells, $CD34^+CD38^-$ cells and all lineages of CFU were enhanced with the addition of FL. In accordance with previous studies, FL increased CFU-MK and the percentage of $CD61^+CD41^+$ cells in QBSF-60, showing that FL had some positive effects on megakaryocytic progenitors in the present culture system. Comparing the effects of FL on the engraftment of expanded cells into NOD/SCID mice, expanded cells with FL showed a trend of higher percentages of human $CD45^+$ cells and $CD61^+$ cells in the BM of mice. However, the results were not statistically significant possibly due to the small sample size. Further experiments might be required to confirm this finding.

In conclusion, QBSF-60 or to a lesser extent, StemSpan supported the expansion of early stem and progenitor cells as well as committed myeloid and erythroid progenitors while X-Vivo 10 supported the most efficient expansion of megakaryocytic progenitors. In addition, expanded cells cultured with the cytokine combinations TSG and TSFG in QBSF-60 successfully engrafted in NOD/SCID

mice and differentiated into functional cells of myeloid and lymphoid lineages. We also demonstrated a higher dose of TPO and SCF, and the presence of FL enhanced the expansion whereas autologous CB plasma reduced the yields of $CD34^+$ cells and CFU-GEMM. These culture systems might be applicable to clinical expansion in the attempt to reduce the severity of cytopenia.

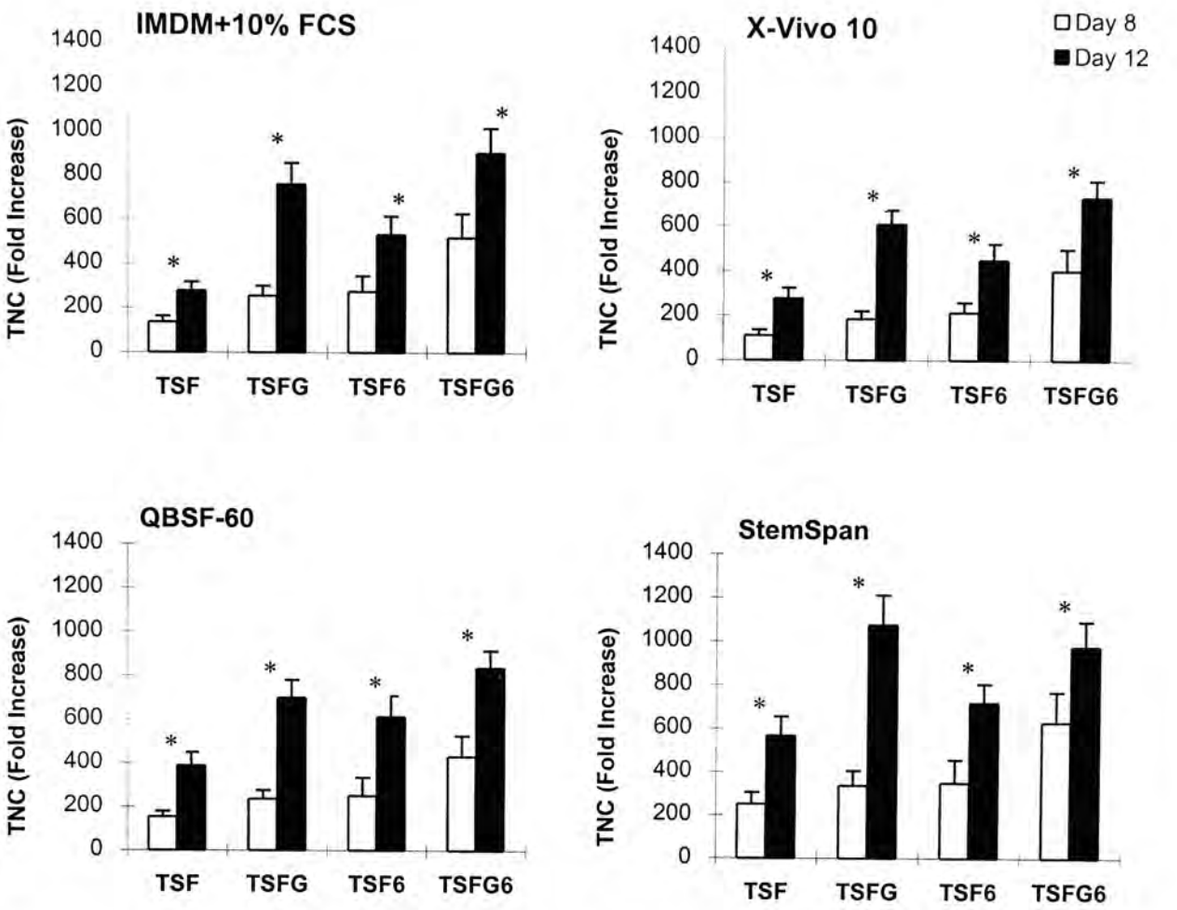


Figure 6.1 Fold Increase of Total Nucleated Cells at Day 8 and Day 12

Enriched $CD34^+$ cells from at 2×10^4/ml were cultured in four media – IMDM + 10% FCS, X-Vivo 10, QBSF-60 and StemSpan, with four cytokine combinations (T – 50 ng/ml TPO, S – 50 ng/ml SCF, F – 80 ng/ml FL, G – 40 ng/ml G-CSF and 6 – 100 ng/ml IL-6) for 12 days. Results were presented as mean ± S.E. TNC – total nucleated cells, n = 10, * $p < 0.05$

Table 6.1 **Expansion of $CD34^+$ Cells and $CD34^+CD38^-$ Cells at Day 8 and Day 12**

Enriched $CD34^+$ cells at 2×10^4/ml were expanded in IMDM + 10% FCS, X-Vivo 10, QBSF-60 or StemSpan in the presence of four cytokine combinations. $CD34^+$ cells and $CD34^+CD38^-$ cells were analyzed by flow cytometry after expansion. The resulting populations were expressed as mean ± S.E. derived from 1 ml of culture at day 0. $n = 10$, * $p < 0.05$

Table 6.1 Expansion of $CD34^+$ Cells and $CD34^+CD38^-$ Cells at Day 8 and Day 12

		$CD34^+$ Cells				$CD34^+CD38^-$ Cells			
		%		$\times 10^5$		%		$\times 10^5$	
Medium	Cytokines	Day 8	Day 12	Day 8	Day 12	Day 8	Day 12	Day 8	Day 12
IMDM + 10% FCS	**TSF**	7.97 ± 1.41*	3.92 ± 1.10*	2.02 ± 0.52	2.61 ± 1.00	1.18 ± 0.35	0.99 ± 0.47	0.33 ± 1.12	0.71 ± 0.42
	TSFG	5.35 ± 1.07*	2.43 ± 0.57*	2.39 ± 0.50	3.19 ± 0.51	1.27 ± 0.34	0.60 ± 0.22	0.60 ± 0.19	0.74 ± 0.20
	TSF6	8.95 ± 1.94	6.11 ± 1.82	4.04 ± 0.79*	5.90 ± 1.75*	2.19 ± 0.59*	1.25 ± 0.29*	1.08 ± 0.45	1.31 ± 0.32
	TSFG6	7.62 ± 1.40*	2.66 ± 0.71*	6.41 ± 1.42	4.30 ± 0.90	1.87 ± 0.35*	0.59 ± 0.20*	1.85 ± 0.49	0.86 ± 0.26
X-Vivo 10	**TSF**	9.37 ± 1.77	6.08 ± 0.99	1.82 ± 0.44*	3.51 ± 1.00*	2.62 ± 0.61	1.58 ± 0.35	0.44 ± 0.09	0.79 ± 0.21
	TSFG	7.74 ± 1.37*	4.31 ± 0.49*	2.53 ± 0.44*	5.37 ± 0.93*	2.36 ± 0.70*	0.68 ± 0.18*	0.66 ± 0.15	0.72 ± 0.17
	TSF6	13.3 ± 2.08*	6.61 ± 1.11*	4.02 ± 0.96	4.96 ± 0.37	4.09 ± 1.01*	1.74 ± 0.38*	0.96 ± 0.22*	1.23 ± 0.25*
	TSFG6	9.37 ± 1.23*	3.38 ± 0.26*	7.01 ± 1.83	4.82 ± 0.61	2.23 ± 0.53*	0.88 ± 0.17*	1.57 ± 0.47	1.18 ± 0.18
QBSF-60	**TSF**	14.5 ± 2.82	9.94 ± 1.97	4.38 ± 1.23*	7.91 ±2.03*	5.20 ± 1.17	3.15 ± 0.60	1.54 ± 0.46*	2.26 ± 0.56*
	TSFG	11.9 ± 1.97*	6.79 ± 0.95*	4.98 ± 0.73*	8.69 ± 1.00*	3.48 ± 0.75	1.86 ± 0.42	1.51 ± 0.33*	2.10 ± 0.49*
	TSF6	19.1 ± 2.69	12.7 ± 2.24	7.82 ± 1.26*	11.5 ± 1.28*	5.53 ± 1.17	5.10 ± 0.88	1.78 ± 0.49*	4.65 ± 0.71*
	TSFG6	13.2 ± 1.99*	6.93 ± 0.48*	11.4 ± 3.12	11.3 ± 1.02	3.13 ± 0.59	2.02 ± 0.40	3.04 ± 0.95*	2.84 ± 0.47*
StemSpan	**TSF**	8.55 ± 1.77	5.91 ± 0.82	3.82 ± 0.91*	7.07 ± 1.20*	2.33 ± 0.68	1.28 ± 0.23	1.00 ± 0.31	1.18 ± 0.51
	TSFG	4.15 ± 0.97	2.82 ± 0.65	2.69 ± 0.96*	6.04 ± 1.70*	1.82 ± 0.64*	0.67 ± 0.16*	0.80 ± 0.20	0.88 ± 0.25
	TSF6	7.58 ± 1.04*	4.41 ± 0.49*	3.72 ± 0.63	5.64 ± 0.66	2.32 ± 0.74	1.33 ± 0.19	0.86 ± 0.34*	1.90 ± 0.45*
	TSFG6	9.92 ± 1.61*	3.42 ± 0.19*	9.98 ± 1.65	6.69 ± 0.90	1.99 ± 0.37*	0.91 ± 0.24*	2.44 ± 0.72	1.67 ± 0.33

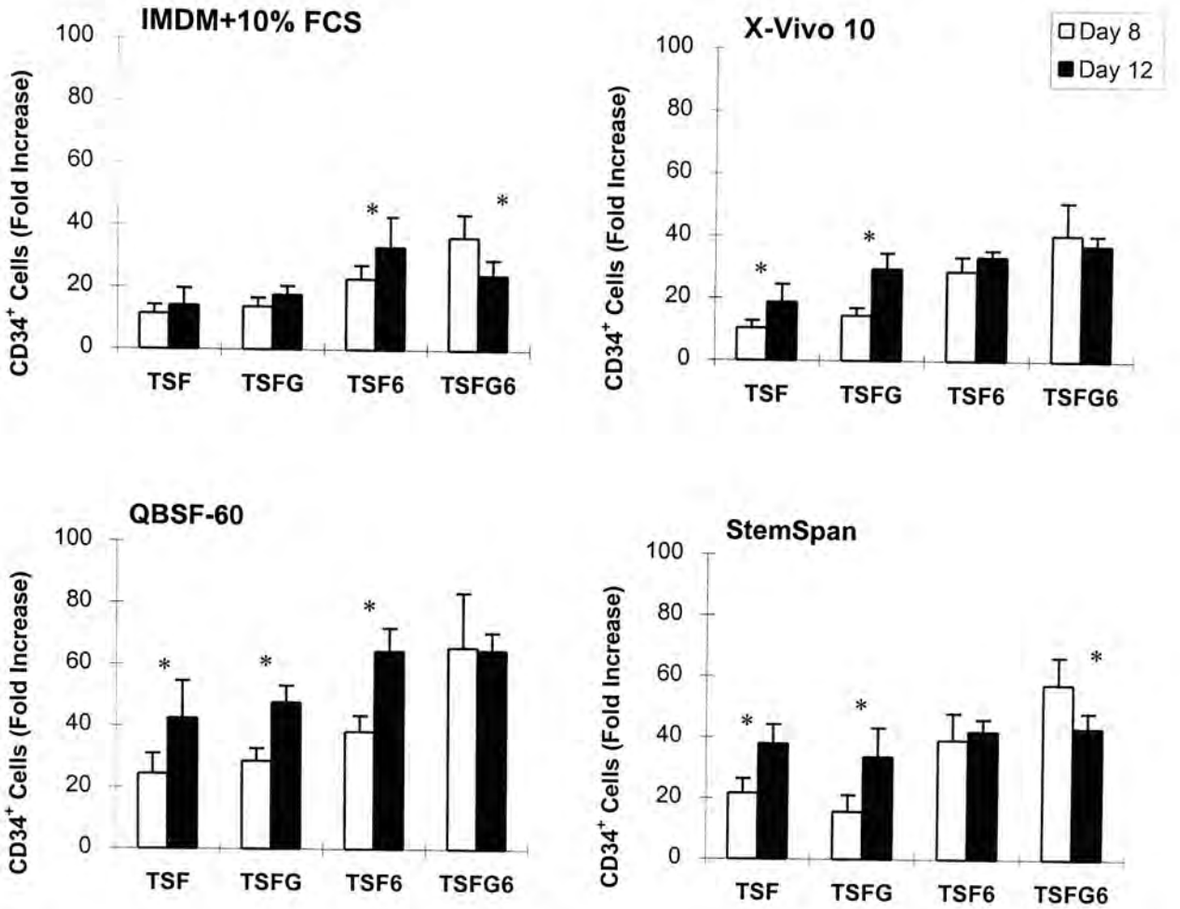


Figure 6.2 Fold Increase of $CD34^+$ Cells at Day 8 and Day 12

The yields of $CD34^+$ cells after expansion were analyzed by flow cytometry. An increase in the fold increase of $CD34^+$ cells was seen at day 12 of culture, except for the cytokine combination TSFG6. Fold increases of $CD34^+$ cells at day 12 were less than those at day 8 in IMDM, X-Vivo 10 and StemSpan with TSFG6. n = 10, * $p < 0.05$

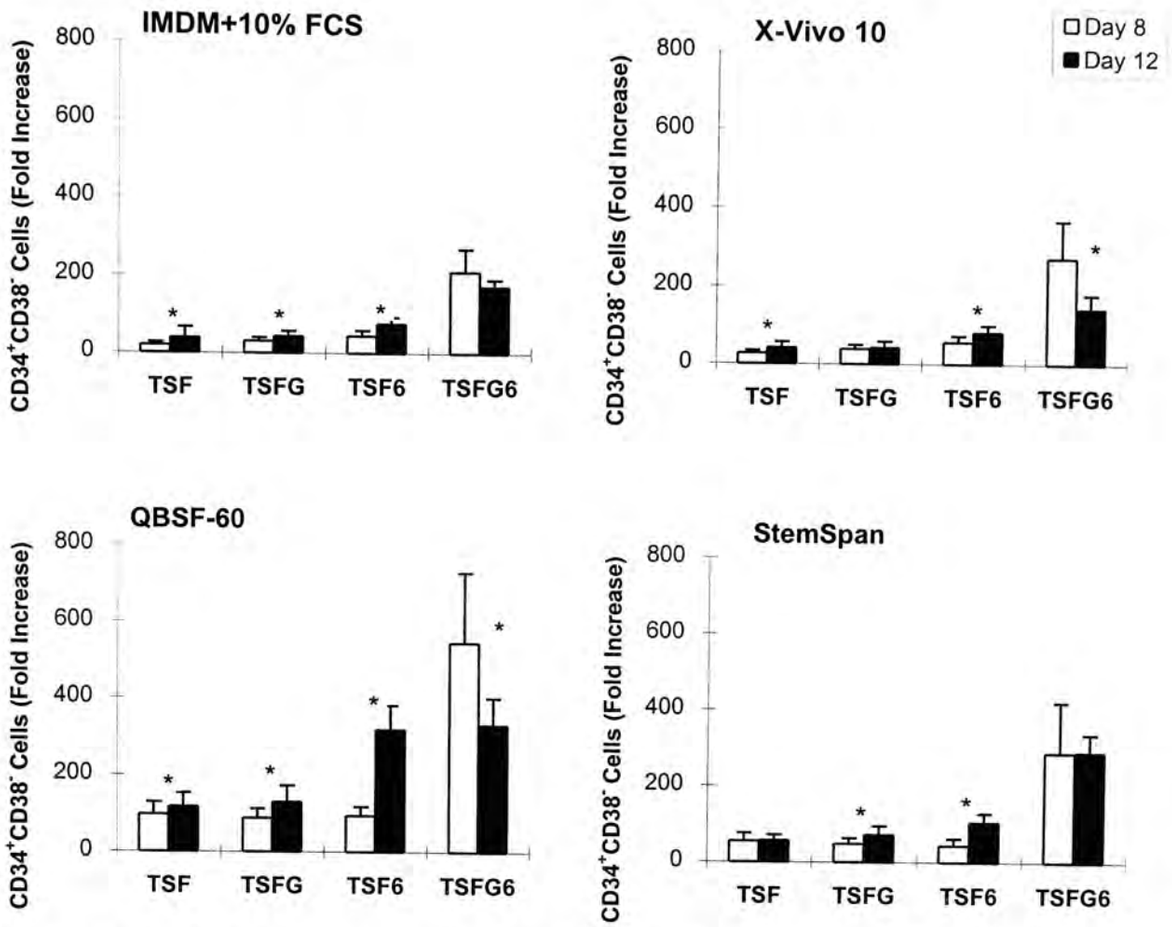


Figure 6.3 Fold Increase of $CD34^+CD38^-$ Cells at Day 8 and Day 12

$CD34^+CD38^-$ cells after expansion were analyzed by flow cytometry. An increase in fold increase of $CD34^+CD38^-$ cells was seen at day 12 of culture, except for the cytokine combination TSFG6. n = 10, * $p < 0.05$

Table 6.2 **Fold Increase of Different Lineages of Colony-Forming Units at Day 8 and Day 12**

Enriched $CD34^+$ cells at 2×10^4/ml were expanded in IMDM + 10% FCS, X-Vivo 10, QBSF-60 or StemSpan in the presence of four cytokine combinations. Fold increases of CFU production were higher at day 12 than those at day 8. Results were expressed as mean ± S.E. n = 10, * $p < 0.05$

Total CFU = CFU-GM + BFU/CFU-E + CFU-GEMM

Table 6.2 Fold Increase of Different Lineages of Colony-forming Units at Day 8 and Day 12

		Total CFU		CFU-GM		BFU/CFU-E		CFU-GEMM	
Medium	Cytokines	Day 8	Day 12	Day 8	Day 12	Day 8	Day 12	Day 8	Day 12
IMDM + 10% FCS	**TSF**	62.1 ± 11.5*	104 ± 11.8*	67.3 ± 10.5*	163 ± 36.4*	38.9 ± 8.63	29.4 ± 6.10	10.5 ± 2.09	7.20 ± 2.17
	TSFG	185 ± 41.6*	230 ± 24.9*	172 ± 27.5*	328 ± 49.7*	94.0 ± 19.4	102 ± 21.7	33.1 ± 5.22*	63.0 ± 16.1*
	TSF6	149 ± 34.7*	190 ± 46.1*	214 ± 64.0*	282 ± 95.3*	97.1 ± 20.8	76.5 ± 33.8	29.7 ± 7.37	24.8 ± 8.18
	TSFG6	179 ± 40.6*	250 ± 46.0*	248 ± 70.5*	417 ± 99.7*	78.4 ± 30.2*	123 ± 59.5*	34.8 ± 6.61*	56.7 ± 13.0*
X-Vivo 10	**TSF**	52.0 ± 16.0*	88.8 ± 14.4*	54.5 ± 11.3*	119 ± 20.9*	26.4 ± 7.72	38.1 ± 9.53	10.7 ± 4.16	9.84 ± 4.02
	TSFG	104 ± 26.8*	174 ± 14.9*	104 ± 12.6*	278 ± 47.8*	53.3 ± 8.70*	99.9 ± 27.1*	24.3 ± 6.30*	41.3 ± 13.3*
	TSF6	101 ± 18.7*	134 ± 20.6*	129 ± 30.4*	164 ± 46.6*	69.3 ± 13.0	58.7 ± 22.1	23.3 ± 6.79	15.0 ± 3.86
	TSFG6	108 ± 15.2*	177 ± 13.2*	155 ± 21.7*	211 ± 56.0*	43.1 ± 7.49	52.4 ± 12.7	14.6 ± 7.65	10.4 ± 1.85
QBSF-60	**TSF**	101 ± 19.2 *	137 ± 15.4*	117 ± 27.5*	199 ± 30.3*	60.8 ± 11.7	53.4 ± 16.0	29.1 ± 6.26	23.6 ± 15.2
	TSFG	189 ± 45.4*	273 ± 31.1*	177 ± 21.7*	370 ± 51.4*	107 ± 23.8*	144 ± 48.5*	40.4 ± 5.89*	164 ± 56.9*
	TSF6	188 ± 52.4*	249 ± 65.4*	229 ± 85.0*	304 ± 125*	123 ± 41.0	124 ± 61.7	53.4 ± 10.7	48.0 ± 15.4
	TSFG6	166 ± 19.6*	243 ± 21.0*	231 ± 20.5*	407 ± 43.2*	80.6 ± 11.7*	127 ± 25.1*	107 ± 25.4*	248 ± 71.7*
StemSpan	**TSF**	88.2 ± 20.0*	138 ± 20.1*	131 ± 42.2*	128 ± 40.0*	52.5 ± 20.6*	69.2 ± 21.0*	10.1 ±2.34*	16.5 ± 6.75*
	TSFG	180 ± 45.2*	271 ± 41.8*	144 ± 17.0*	351 ± 80.0*	59.9 ± 16.1*	126 ± 48.5*	28.9 ± 7.61*	102 ± 36.9*
	TSF6	157 ± 33.1*	193 ± 47.4*	221 ± 72.6*	253 ± 103*	103 ± 17.7	63.1 ± 26.1	46.6 ± 8.15	35.9 ± 12.3
	TSFG6	178 ± 32.4*	250 ± 38.9*	273 ± 90.0*	358 ± 122*	79.6 ± 15.8*	91.0 ± 25.1*	43.0 ± 10.2*	130 ± 48.7*

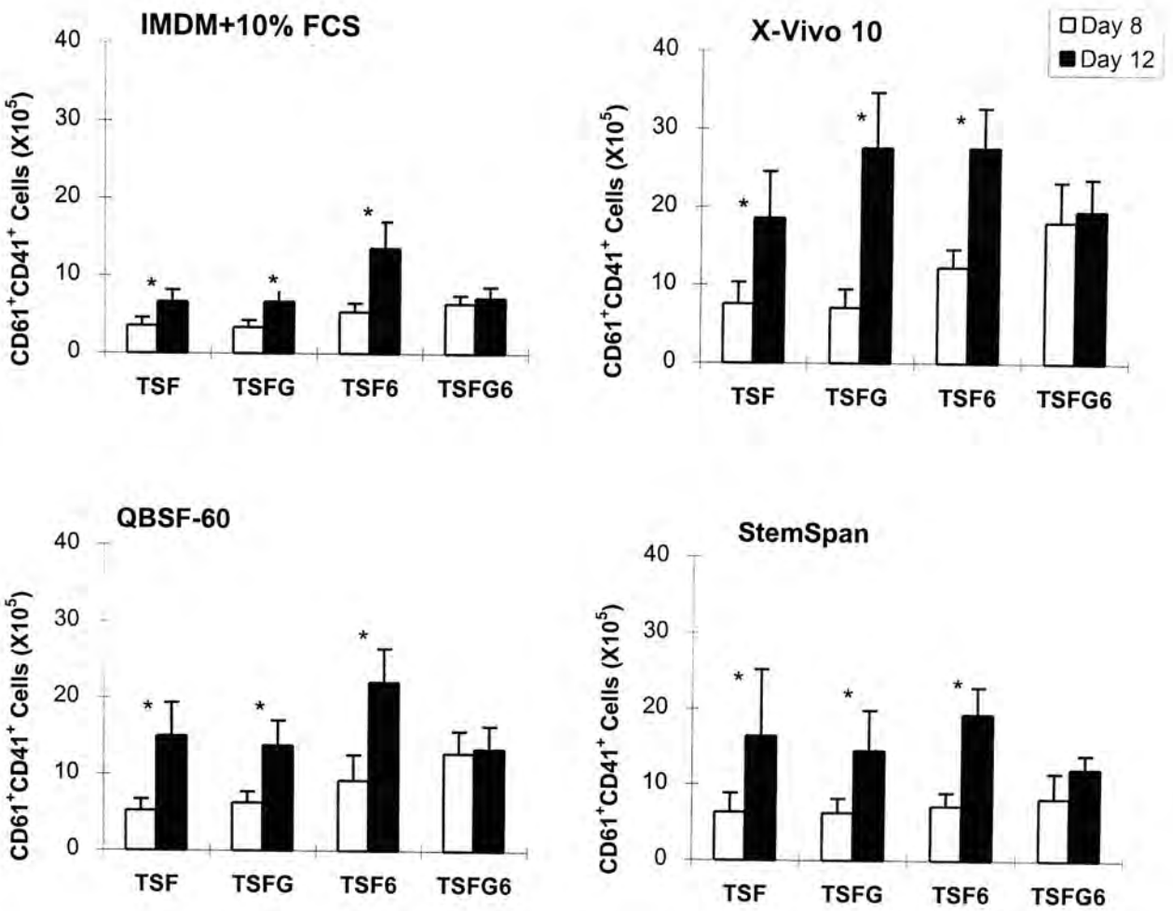


Figure 6.4 Expansion of $CD61^+CD41^+$ Cells at Day 8 and Day 12

Megakaryocytic $CD61^+CD41^+$ cells after expansion were analyzed by flow cytometry. The yields of $CD61^+CD41^+$ cells were significantly higher at day 12 than those at day 8 in the presence of cytokine combinations TSF, TSFG and TSF6 in IMDM, X-Vivo 10, QBSF-60 and StemSpan. n = 10, * $p < 0.05$

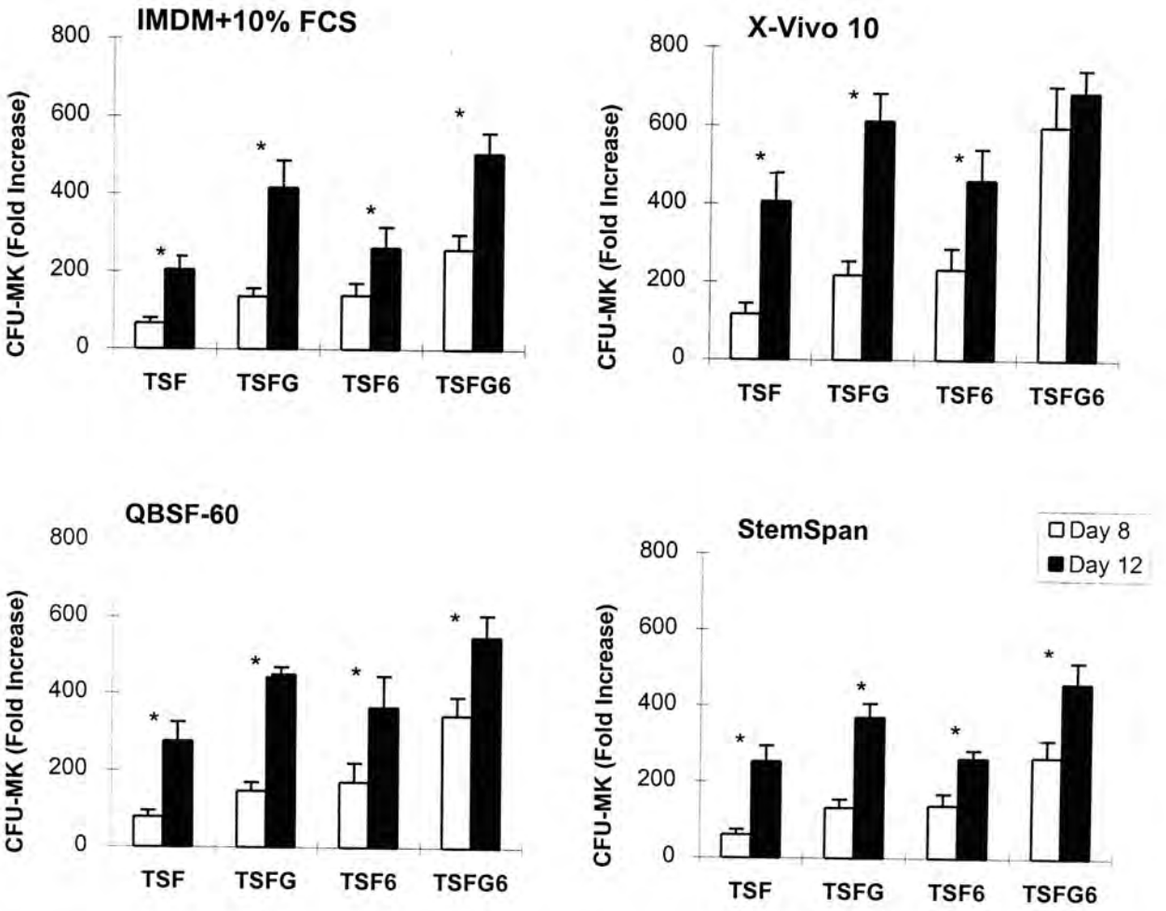


Figure 6.5 Fold Increase of CFU-MK at Day 8 and Day 12

Expanded cells at 3×10^3/ml were seeded in the plasma clot system for CFU-MK assay. The fold increases of CFU-MK at day 12 were significantly higher when compared to those at day 8. n = 10, * $p < 0.05$

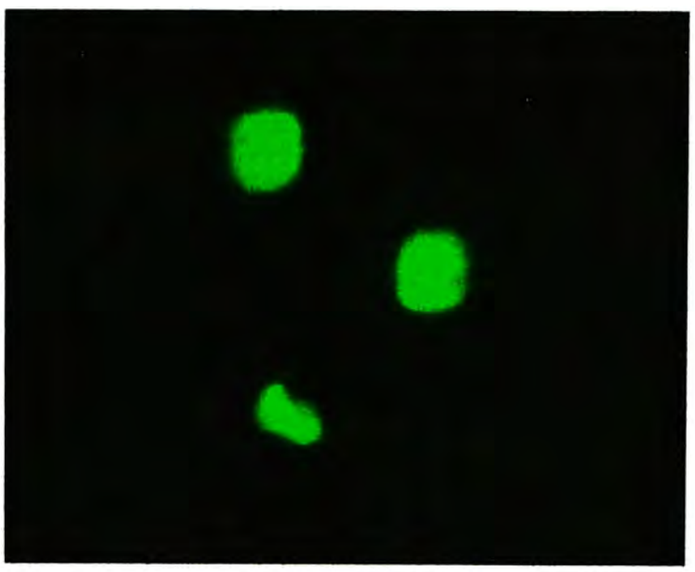


Figure 6.6 Immunofluoresence Staining of CFU-MK

Expanded cells were harvested for CFU-MK assay using the plasma clot system. After 12 days of incubation, colonies were stained with CD61-FITC. Three or more CD61-positive cells were scored as a CFU-MK colony. Photograph was taken at 50× original magnification.

Table 6.3 **Effects of Various Media and Cytokine Combinations on the *Ex Vivo* Expansion of Cord Blood $CD34^+$ Cells**

Enriched $CD34^+$ cells at 2×10^4/ml were expanded in the presence of four cytokine combinations in four media for 12 days. The resulting populations derived from 1 ml of culture at day 0 were expressed as mean ± S.E. n = 10, * $p < 0.05$

Table 6.3 Effects of Various Media and Cytokine Combinations on *Ex vivo* expansion of Cord Blood $CD34^+$ cells

	IMDM + 10% FCS				X-Vivo 10				QBSF-60				StemSpan SFEM			
	TSF	TSFG	TSF6	TSFG6	TSF	TSFG	TSF6	TSFG6	TSF	TSFG	TSF6	TSFG6	TSF	TSFG	TSF6	TSFG6
Total Nucleated Cells ($\times 10^6$)	5.57 ± 0.78	15.2 ± 1.95	10.7 ± 1.68	18.1 ± 2.27	5.51 ± 0.96	12.2 ± 1.28	9.04 ± 1.49	14.6 ± 1.49	7.77 ± 1.20	14.0 ± 1.61	12.3 ± 1.93	16.7 ± 1.59	11.3 ± 1.75	21.5 ± 2.71	14.4 ± 1.71	19.5 ± 2.31
$CD34^+$ cells (%)	3.92 ± 1.10	2.43 ± 0.57	6.11 ± 1.82	2.66 ± 0.71	6.08 ± 0.99	4.31 ± 0.49	6.61 ± 1.11	3.38 ± 0.26	9.94 ± 1.97	6.79 ± 0.95	12.7 ± 2.24	6.93 ± 0.48	5.91 ± 0.82	2.82 ± 0.65	4.41 ± 0.49	3.42 ± 0.19
$CD34^+$ Cells ($\times 10^5$)	2.61 ± 1.00	3.19 ± 0.51	5.90 ± 1.75	4.30 ± 0.90	3.51 ± 1.00	5.37 ± 0.93	4.96 ± 0.37	4.82 ± 0.61	7.91 ± 2.03	8.69 ± 1.00	11.5 ± 1.28	11.4 ± 1.02	7.07 ± 1.20	6.04 ± 1.70	5.64 ± 0.66	6.69 ± 0.90
$CD34^+38^-$ Cells (%)	0.99 ± 0.47	0.60 ± 0.22	1.25 ± 0.29	0.59 ± 0.20	1.58 ± 0.35	0.68 ± 0.18	1.74 ± 0.38	0.88 ± 0.17	3.15 ± 0.60	1.86 ± 0.42	5.10 ± 0.88	2.02 ± 0.40	1.28 ± 0.23	0.67 ± 0.16	1.33 ± 0.19	0.91 ± 0.24
$CD34^+38^-$ Cells ($\times 10^4$)	7.10 ± 4.20	7.40 ± 2.00	12.3 ± 3.20	8.60 ± 2.60	7.90 ± 2.10	7.18 ± 1.74	13.1 ± 2.45	11.8 ± 1.84	22.6 ± 5.60	21.0 ± 4.88	46.5 ± 7.11	30.4 ± 4.72	11.8 ± 5.13	8.00 ± 2.50	19.0 ± 4.46	16.7 ± 3.32
Total CFU ($\times 10^5$)	1.91 ± 0.21	7.59 ± 1.00	4.50 ± 1.17	4.99 ± 0.75	1.68 ± 0.30	5.06 ± 0.84	2.81 ± 0.53	4.71 ± 0.41	2.48 ± 0.40	8.12 ± 1.73	5.14 ± 1.58	6.46 ± 0.63	2.95 ± 0.58	8.73 ± 1.36	4.34 ± 1.24	6.52 ± 0.96
CFU-GM ($\times 10^5$)	1.42 ± 0.22	4.42 ± 0.76	2.95 ± 0.97	4.62 ± 0.88	1.14 ± 0.22	2.64 ± 0.33	1.79 ± 0.46	2.22 ± 0.53	1.87 ± 0.30	3.63 ± 0.45	3.20 ± 1.27	4.96 ± 0.56	1.70 ± 0.42	3.85 ± 0.82	2.84 ± 1.00	3.60 ± 1.13
BFU/CFU-E ($\times 10^4$)	2.57 ± 0.75	7.49 ± 1.92	5.50 ± 2.06	8.37 ± 3.53	3.31 ± 0.86	6.43 ± 1.31	4.28 ± 1.39	4.13 ± 0.80	4.34 ± 1.21	9.09 ± 1.82	8.34 ± 3.73	1.04 ± 0.82	6.85 ± 1.31	6.66 ± 1.34	4.81 ± 1.62	7.49 ± 2.46
CFU-GEMM ($\times 10^4$)	0.46 ± 0.14	2.65 ± 0.65	1.22 ± 0.46	2.60 ± 0.70	0.64 ± 0.19	1.77 ± 0.53	0.75 ± 0.19	0.51 ± 0.08	1.54 ± 0.63	6.97 ± 0.23	2.62 ± 0.81	10.1 ± 2.81	1.10 ± 0.27	4.52 ± 1.45	2.07 ± 0.48	5.42 ± 1.88
$CD61^+41^+$ Cells ($\times 10^5$)	6.60 ± 1.49	6.74 ± 1.98	13.6 ± 3.52	6.48 ± 1.09	18.6 ± 6.00	27.9 ± 7.11	27.6 ± 5.10	19.5 ± 4.21	15.0 ± 4.38	13.8 ± 3.26	22.1 ± 4.35	13.4 ± 2.90	16.4 ± 8.87	14.5 ± 5.30	19.3 ± 3.60	8.20 ± 1.87
CFU-MK ($\times 10^4$)	6.40 ± 1.11	13.4 ± 2.11	9.40 ± 1.90	20.5 ± 2.12	12.7 ± 2.88	21.0 ± 2.06	16.5 ± 2.88	27.7 ± 2.29	8.60 ± 1.60	15.4 ± 2.70	13.1 ± 2.85	22.2 ± 2.31	7.90 ± 1.26	11.0 ± 1.31	9.42 ± 0.70	18.6 ± 2.33

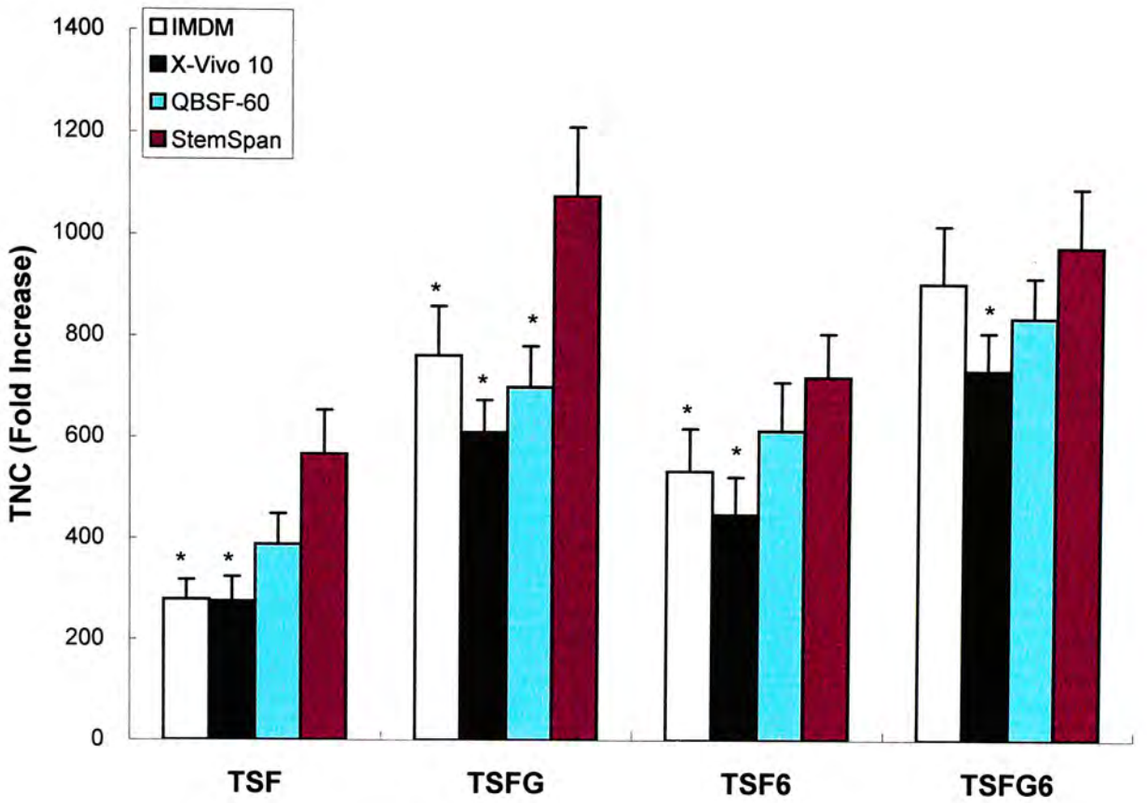


Figure 6.7 **Effects of Culture Media on the Fold Increase of Total Nucleated Cells**

Enriched $CD34^+$ cells at 2×10^4/ml were cultured in four media – IMDM + 10%FCS, X-Vivo 10, QBSF-60 and StemSpan in the presence of four cytokine combinations. After 12 days of culture, StemSpan supported significantly higher expansion of total nucleated cells (TNC) than IMDM + 10% FCS, X-Vivo 10 and QBSF-60. n = 10, * $p < 0.05$ (StemSpan vs other media)

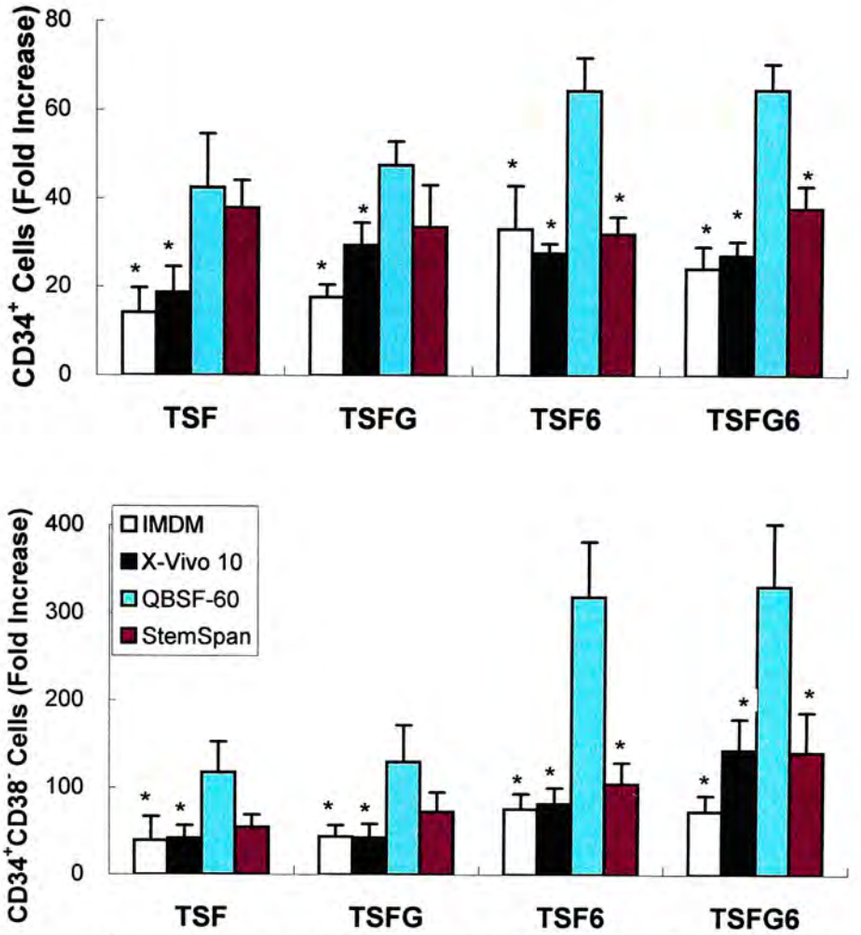


Figure 6.8 Effects of Culture Media on the Fold Increase of $CD34^+$ Cells and $CD34^+CD38^-$ Cells

Expanded cells were harvested for flow cytometry analysis of $CD34^+$ cells and $CD34^+CD38^-$ cells. The fold increases of both $CD34^+$ and $CD34^+CD38^-$ cells were the highest when cells were cultured in QBSF-60. n = 10, * $p < 0.05$ (QBSF-60 vs other media)

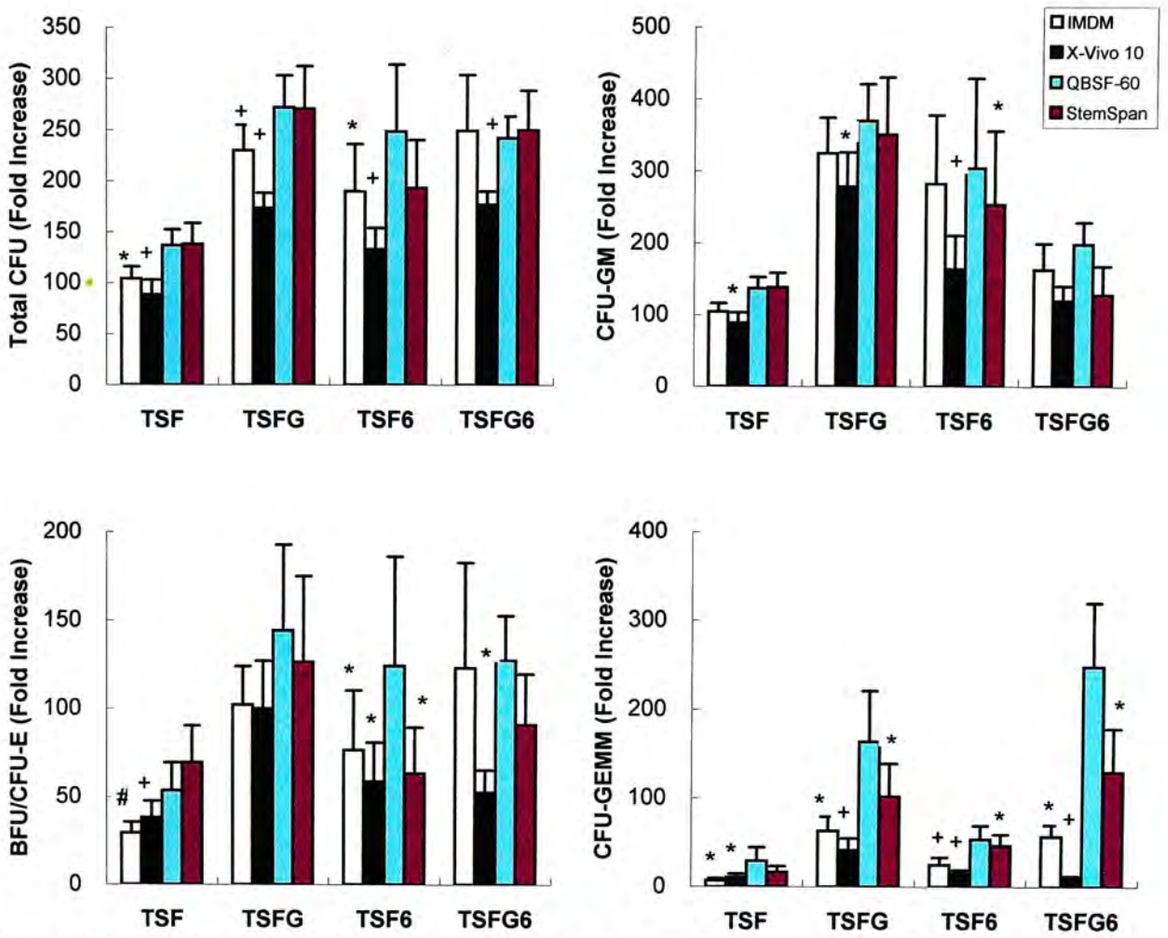


Figure 6.9 Effects of Culture Media on the Fold Increase of CFU

The colony-forming capacity of expanded cells to different lineages of hematopoietic cells was assayed using methylcellulose system. * and # represented significant differences were observed in the fold increases of CFU in QBSF-60 and StemSpan, respectively when compared to those in that particular medium. + represented both QBSF-60 and StemSpan supported significantly higher fold increases of CFU ($p < 0.05$). $n = 10$

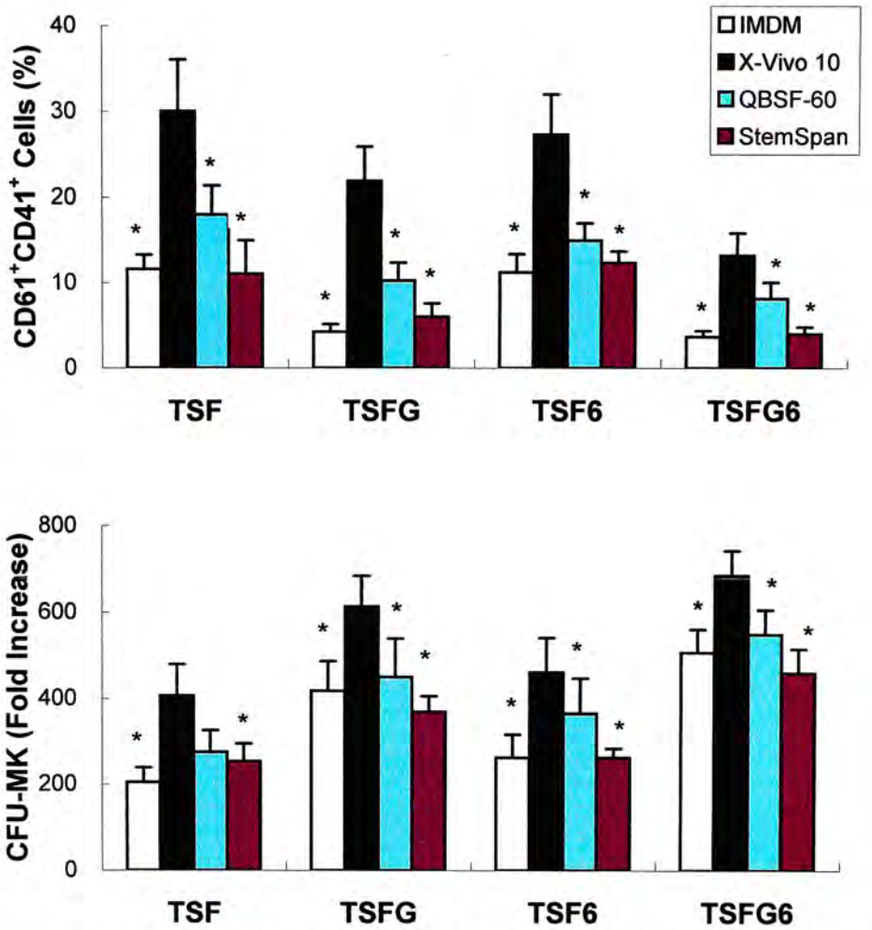


Figure 6.10 Effects of Culture Media on $CD61^+CD41^+$ Cells and CFU-MK

The percentages of $CD61^+41^+$ cells were analyzed by flow cytometry. CFU-MK was assayed using the plasma clot system. Results demonstrated that the highest percentages of $CD61^+41^+$ cells and fold increase of CFU-MK were observed in cultures with X-Vivo 10 (* $p < 0.05$). $n = 10$

Table 6.4 Effects of Autologous Cord Blood Plasma on the Expansion of Total Nucleated Cells in Various Media and Cytokine Combinations

Total Nucleated Cells ($\times 10^6$)	TSF		TSFG		TSF6		TSFG6	
	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)
IMDM	7.34 ± 1.33*	10.2 ± 1.82*	12.9 ± 4.80	15.0 ± 2.99	10.1 ± 2.49	11.2 ± 1.67	13.7 ± 1.78	15.6 ± 2.65
X-Vivo 10	4.39 ± 0.88*	10.9 ± 2.55*	12.6 ± 3.72*	13.5 ± 5.00*	9.89 ± 1.65*	10.8 ± 2.34*	12.9 ± 1.09*	16.6 ± 2.82*
QBSF-60	9.34 ± 3.12*	11.1 ± 2.98*	13.6 ± 2.16	14.8 ± 4.28	13.1 ± 3.98*	13.7 ± 2.79*	23.7 ± 3.82*	26.0 ± 4.42*
StemSpan	12.1 ± 2.46*	14.4 ± 4.85*	25.1 ± 6.86	26.7 ± 5.76	21.3 ± 5.99*	23.7 ± 3.19*	26.2 ± 2.94*	28.9 ± 6.13*

Enriched $CD34^+$ cells at 2×10^4/ml were cultured in IMDM, X-Vivo 10, QBSF-60, StemSpan in the presence or absence of autologous CB plasma for 12 days. (-) represented the absence of autologous CB plasma, while (+) meant 10% autologous plasma was added. Results were presented as mean ± S.E. n = 6, * $p < 0.05$

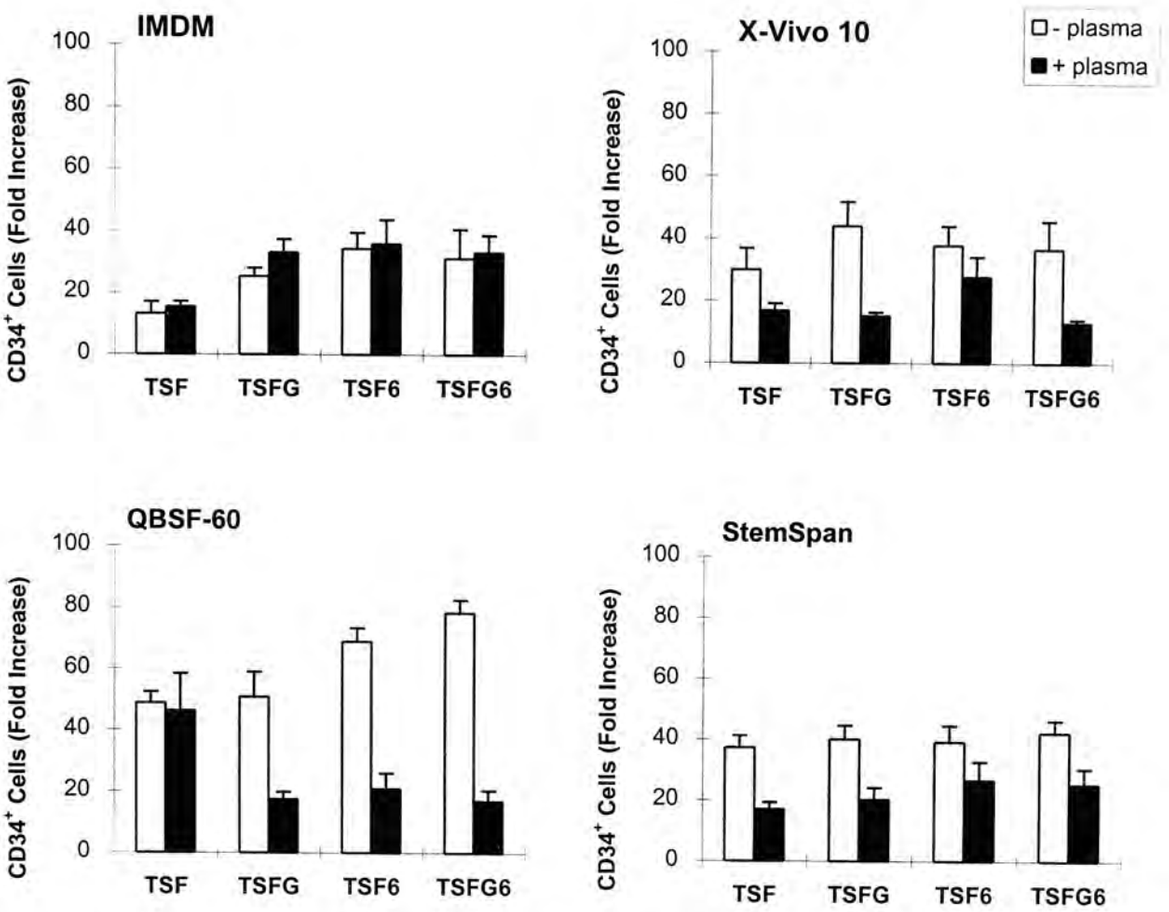


Figure 6.11 Effects of Autologous Cord Blood Plasma on the Fold Increase of $CD34^+$ Cells

$CD34^+$ cells were analyzed by flow cytometry after 12 days of expansion. No significant difference in the fold increase of $CD34^+$ cell was observed when FCS was replaced by autologous plasma in IMDM cultures. In cultures with X-Vivo 10, QBSF-60 and StemSpan, the addition of autologous plasma significantly reduced the fold increases of $CD34^+$ cells. $n = 6$, * $p < 0.05$

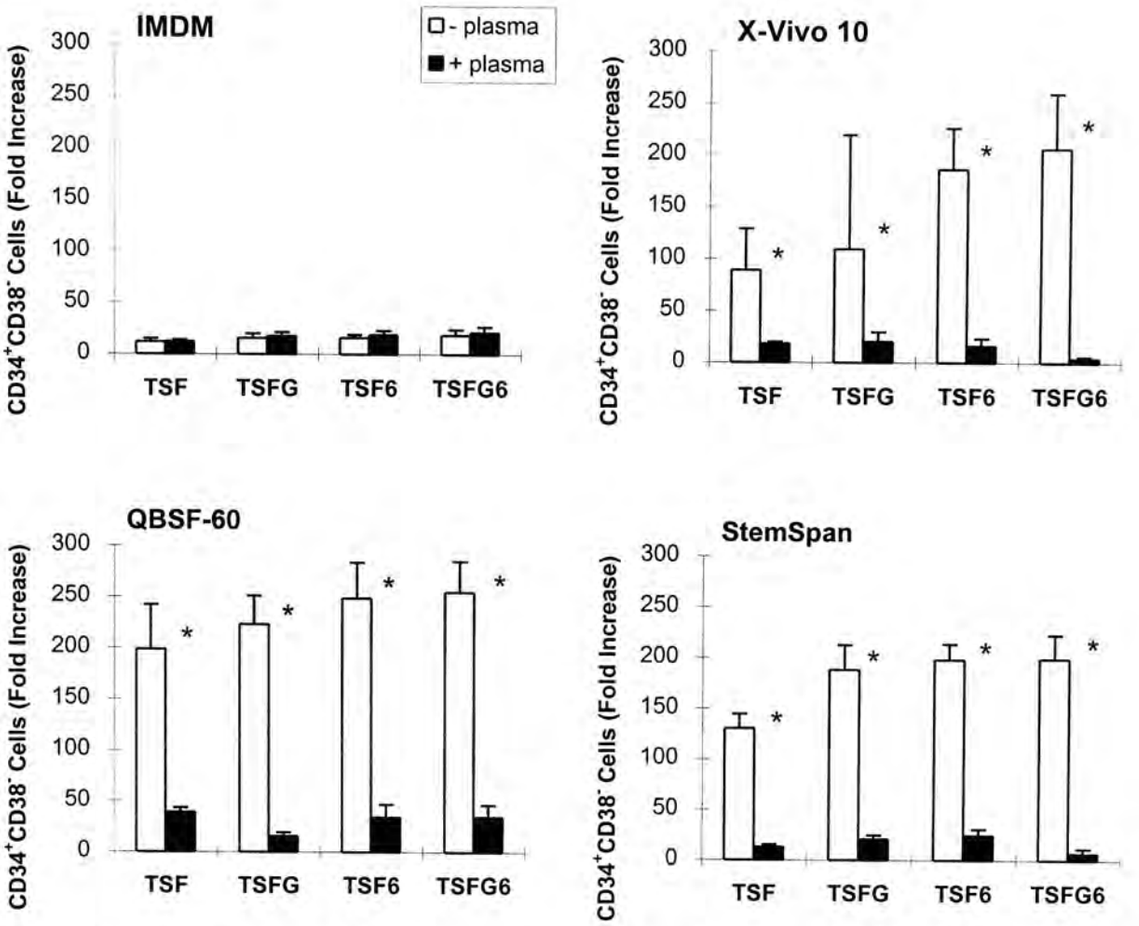


Figure 6.12 Effects of Autologous Cord Blood Plasma on the Fold Increase of $CD34^+CD38^-$ Cells

After 12 days of expansion, cells were harvested for analysis of $CD34^+CD38^-$ cells by flow cytometry. In IMDM cultures, the effect of autologous plasma on the expansion of $CD34^+CD38^-$ cells was similar to that of FCS. However, the addition of autologous plasma to the serum-free media, X-Vivo 10 and QBSF-60 and StemSpan, significantly decreased the fold increases of $CD34^+CD38^-$ cells. n = 6, * $p < 0.05$

Table 6.5 **Effects of Autologous Cord Blood Plasma on the Fold Increase of Different Lineages of Colony-Forming Units**

Enriched $CD34^+$ cells at 2×10^4/ml were expanded in IMDM, X-Vivo 10, QBSF-60 and StemSpan in the presence or absence of autologous CB plasma for 12 days. (-) represented the absence of autologous CB plasma, while (+) meant 10% autologous plasma was added. Results were expressed as mean ± S.E. $n = 6$, * $p < 0.05$

Total CFU = CFU-GM + BFU/CFU-E + CFU-GEMM

Table 6.5 Effects of Autologous Cord Blood Plasma on the Fold Increase of Different Lineages of Colony-forming Units

		Total CFU		CFU-GM		BFU/CFU-E		CFU-GEMM	
Medium	Cytokines	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)
IMDM	**TSF**	155 ± 22.7	117 ± 27.5	202 ± 47.2	242 ± 38.5	53.2 ± 16.8	102 ± 34.7	5.63 ± 1.11	4.88 ± 1.56
	TSFG	225 ± 34.0	177 ± 21.7	630 ± 115	749.3 ± 168	24.6 ± 3.78	24.5 ± 4.40	12.2 ± 5.11	10.3 ± 1.38
	TSF6	305 ± 105	229 ± 85.0	224 ± 72.6	234 ± 27.2	59.8 ± 16.2	73.6 ± 24.3	31.3 ± 20.5	38.4 ± 11.9
	TSFG6	251 ± 32.7	231 ± 20.5	437 ± 66.6	566 ± 70.6	160.1 ± 52.2	260 ± 88.4	127 ± 67.7	130 ± 40.8
X-Vivo 10	**TSF**	199 ± 46.4	180 ± 40.0	182 ± 52.4*	249 ± 35.3*	56.0 ± 15.3*	103 ± 34.7*	10.2 ± 1.03*	6.24 ± 1.27*
	TSFG	203 ± 30.4	213 ± 54.1	136 ± 40.1*	344 ± 171*	28.6 ± 3.51	26.4 ± 4.36	12.7 ± 2.95*	11.12 ± 2.07*
	TSF6	269 ± 57.0	213 ± 44.2	213 ± 73.0*	229 ± 26.2*	58.6 ± 17.0*	74.0 ± 24.0*	66.0 ± 19.7*	42.4 ± 9.76*
	TSFG6	257 ± 34.0	215 ± 35.6	431 ± 67.8*	505 ± 84.4*	167 ± 48.6*	261 ± 88.0*	220 ± 66.2*	130 ± 40.8*
QBSF-60	**TSF**	232 ± 23.3	197 ± 32.9	212 ± 42.3*	286 ± 60.8*	71.3 ± 22.2*	131 ± 49.1*	22.6 ± 16.1*	14.7 ± 10.5*
	TSFG	258 ± 42.6	225 ± 17.7	412 ± 66.6*	530 ± 108*	90.4 ± 37.9*	152 ± 70.7*	119.2 ± 73.7*	93.3 ± 54.1*
	TSF6	260 ± 58.1	238 ± 47.1	449 ± 198*	634 ± 92.7*	181 ± 98.7*	241 ± 131*	59.2 ± 20.5*	26.6 ± 9.24*
	TSFG6	261 ± 31.5	240 ± 52.6	425 ± 39.2*	549 ± 71.2*	131 ± 41.8*	214 ± 70.4*	182 ± 113*	92.9 ± 55.9*
StemSpan	**TSF**	208 ± 40.9	177 ± 41.6	190 ± 33.3*	228 ± 56.0*	65.5 ± 32.1*	105 ± 54.0*	18.5 ± 8.7*	15.2 ± 6.50*
	TSFG	202 ± 31.1	216 ± 53.6	288 ± 76.1	334 ± 81.1	71.8 ± 32.8	119 ± 54.5	88.1 ± 37.7*	43.1 ± 19.8*
	TSF6	276 ± 53.1	215 ± 43.6	350 ± 81.5	442 ± 118	151 ± 65.5	208 ± 94.0	42.0 ± 11.8	30.0 ± 12.0
	TSFG6	257 ± 33.7	246 ± 37.5	321 ± 58.5*	465 ± 98.4*	149 ± 55.8 *	197 ± 24.2*	77.9 ± 19.5*	40.2 ± 5.90*

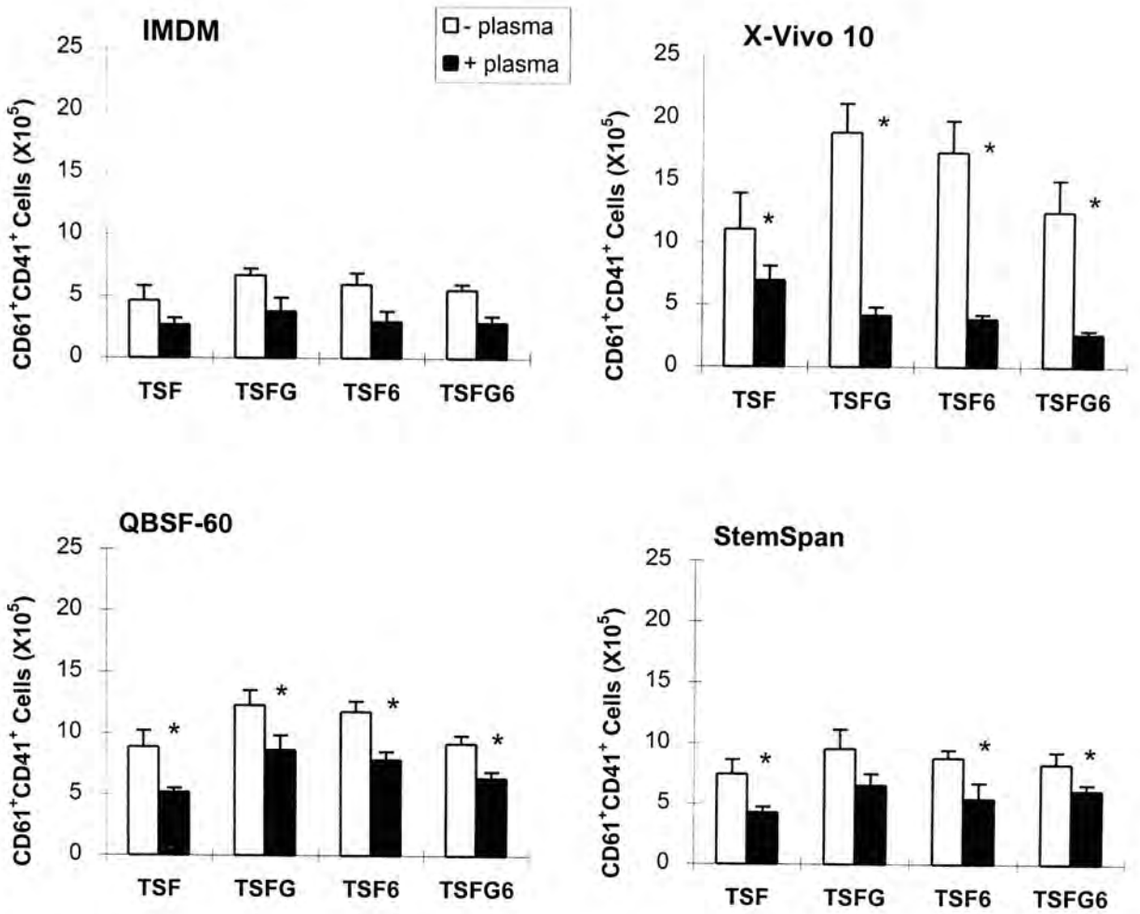


Figure 6.13 Effects of Autologous Cord Blood Plasma on the Expansion of $CD61^+CD41^+$ Cells

The yields of $CD61^+CD41^+$ cells were analyzed by flow cytometry after 12 days of culture in four media with or without 10% autologous plasma. Significant decreases in the yields of $CD61^+CD41^+$ cells were observed in cultures in which autologous plasma was present. n = 6, * $p < 0.05$

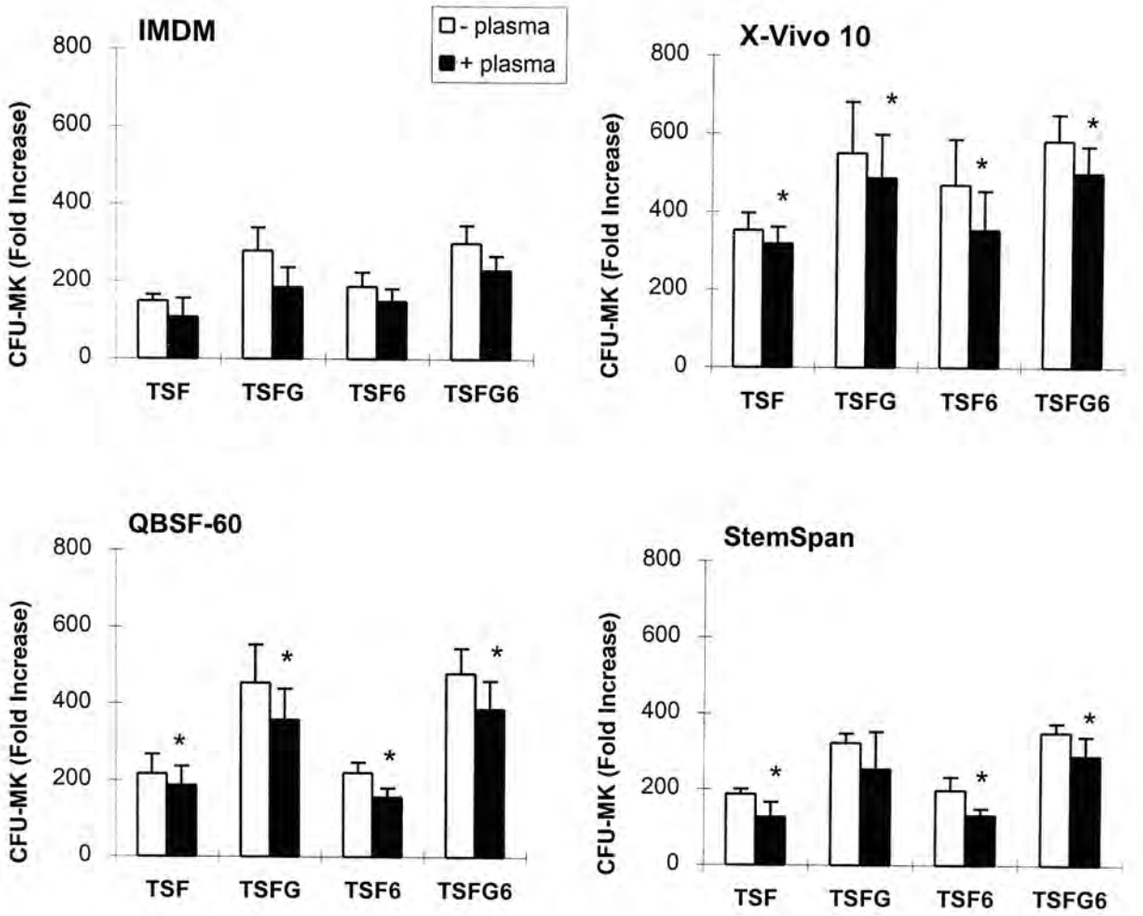


Figure 6.14 Effects of Autologous Cord Blood Plasma on the Fold Increase of CFU-MK

Expanded cells at 3 × 10^3/ml were seeded in the plasma clot system for CFU-MK assay. Similar to the expansion of $CD61^+CD41^+$ cells, the addition of autologous plasma led to significant decreases of fold increases of CFU-MK in the majority of cultures. n = 6, * $p < 0.05$

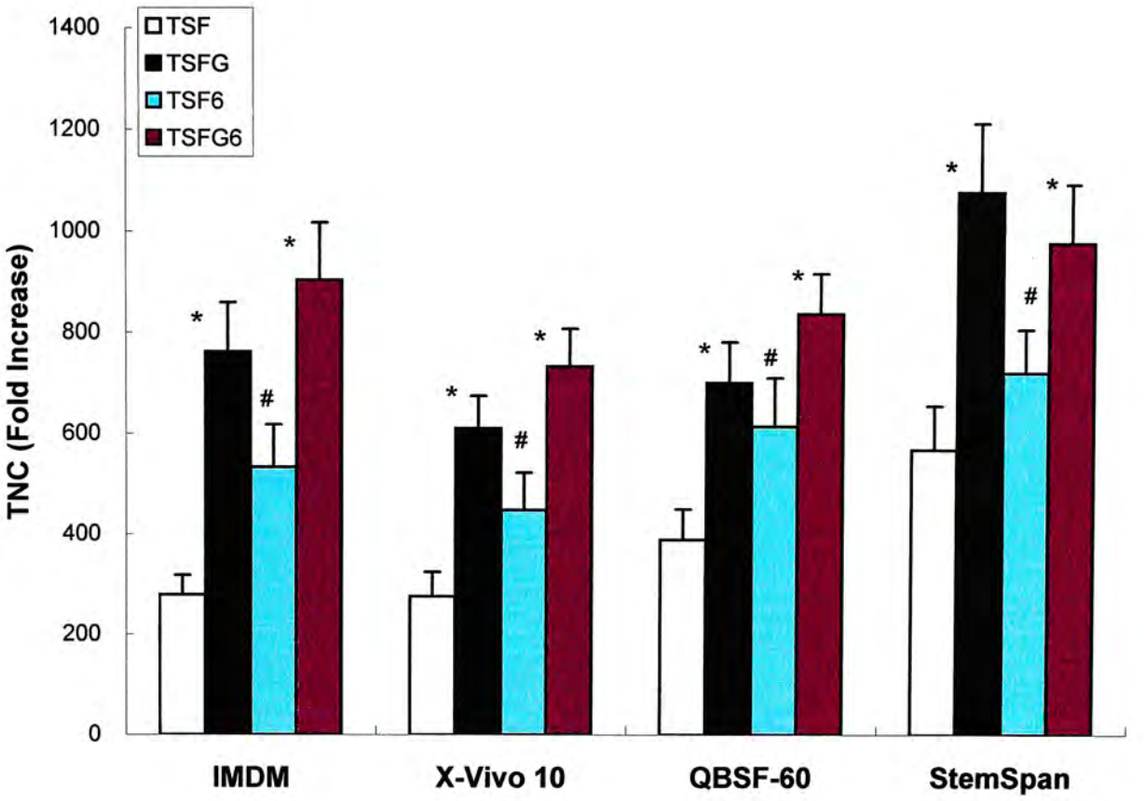


Figure 6.15 Effects of Granulocyte-Colony Stimulating Factor and Interleukin-6 on the Fold Increase of Total Nucleated Cells

Enriched $CD34^+$ cells at 2×10^4/ml were cultured for 12 days in four media with four cytokine combinations. The addition of * G-CSF and # IL-6 to the cultures increased total nucleated cells when compared to the respective treatments without that cytokines ($p < 0.05$). n = 10

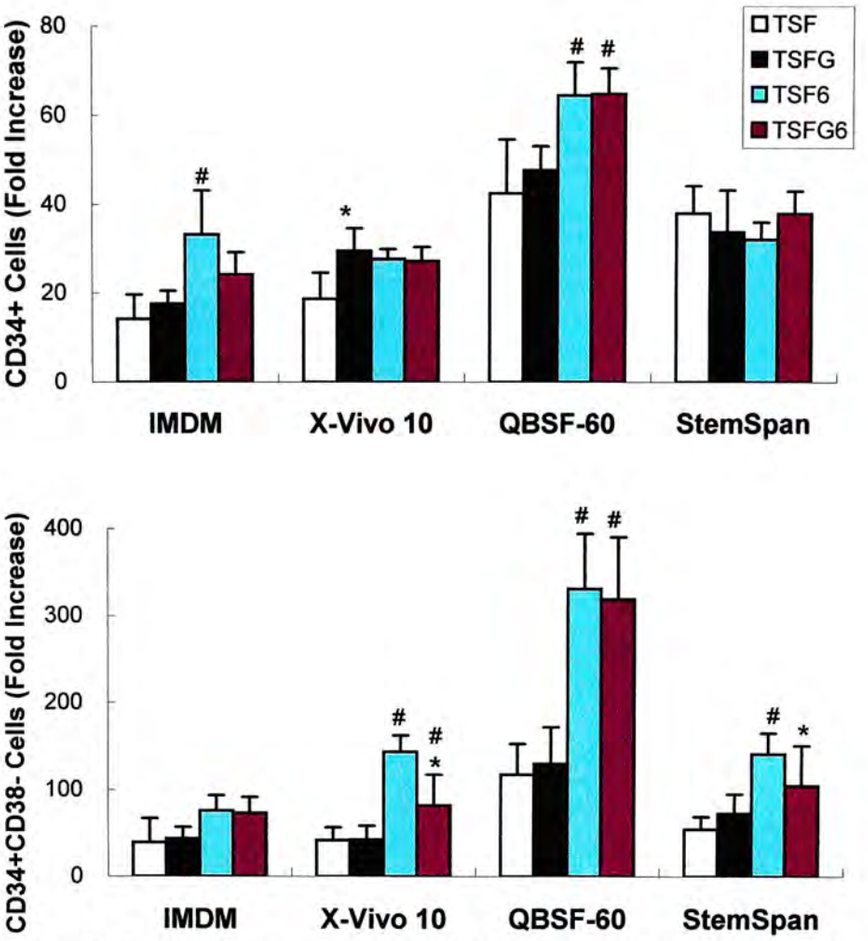


Figure 6.16 Effects of Granulocyte-Colony Stimulating Factor and Interleukin-6 on the Fold Increase of $CD34^+$ Cells and $CD34^+CD38^-$ Cells

$CD34^+$ cells and $CD34^+CD38^-$ cells were analyzed by flow cytometry. Significant differences were observed when * G-CSF or # IL-6 was added ($p < 0.05$). $n = 10$

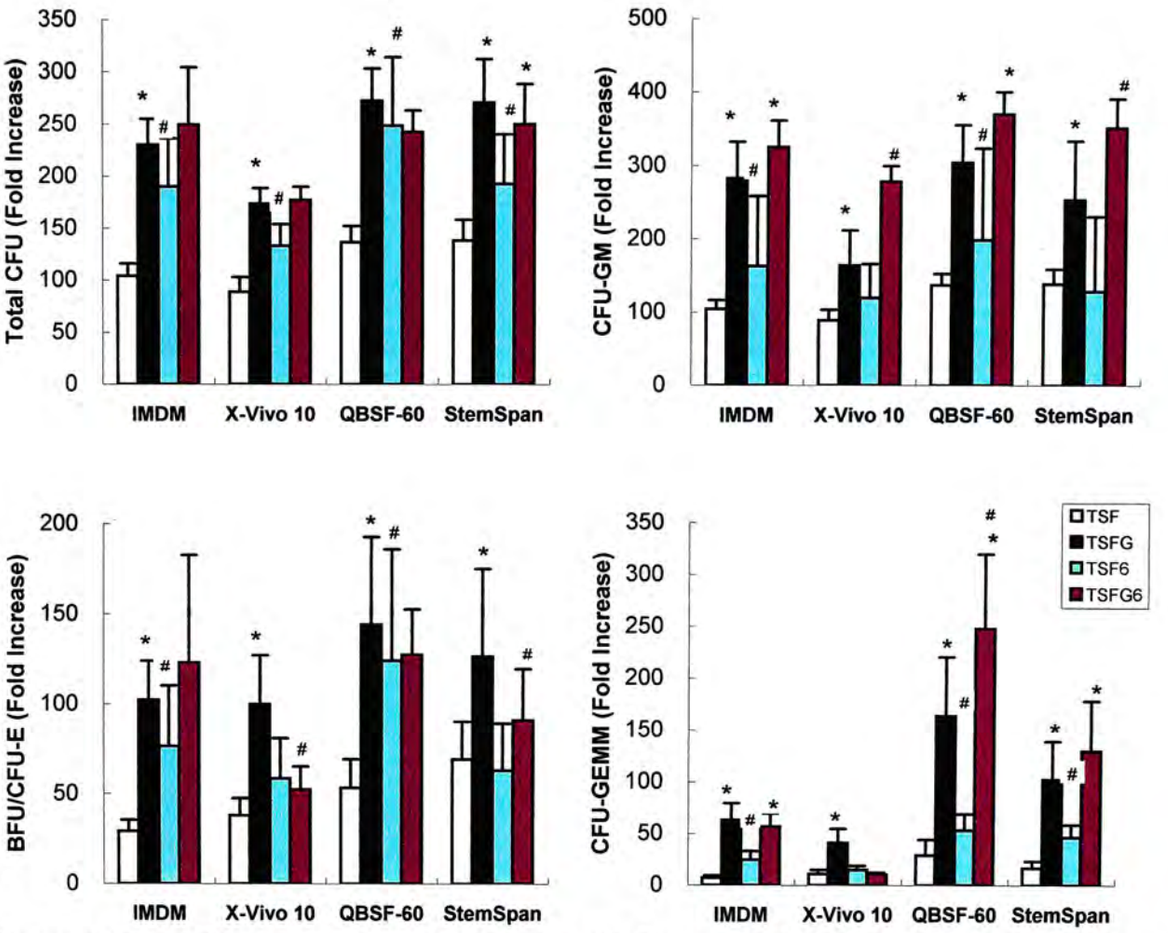


Figure 6.17 Effects of Granulocyte-Colony Stimulating Factor and Interleukin-6 on the Fold Increase of CFU

The fold increases of total CFU (CFU-GM, BFU/CFU-E, CFU-GEMM), CFU-GM, BFU/CFU-E and CFU-GEMM were examined. * (with G-CSF vs without G-CSF) and # (with IL-6 vs without IL-6) $p < 0.05$, $n = 10$

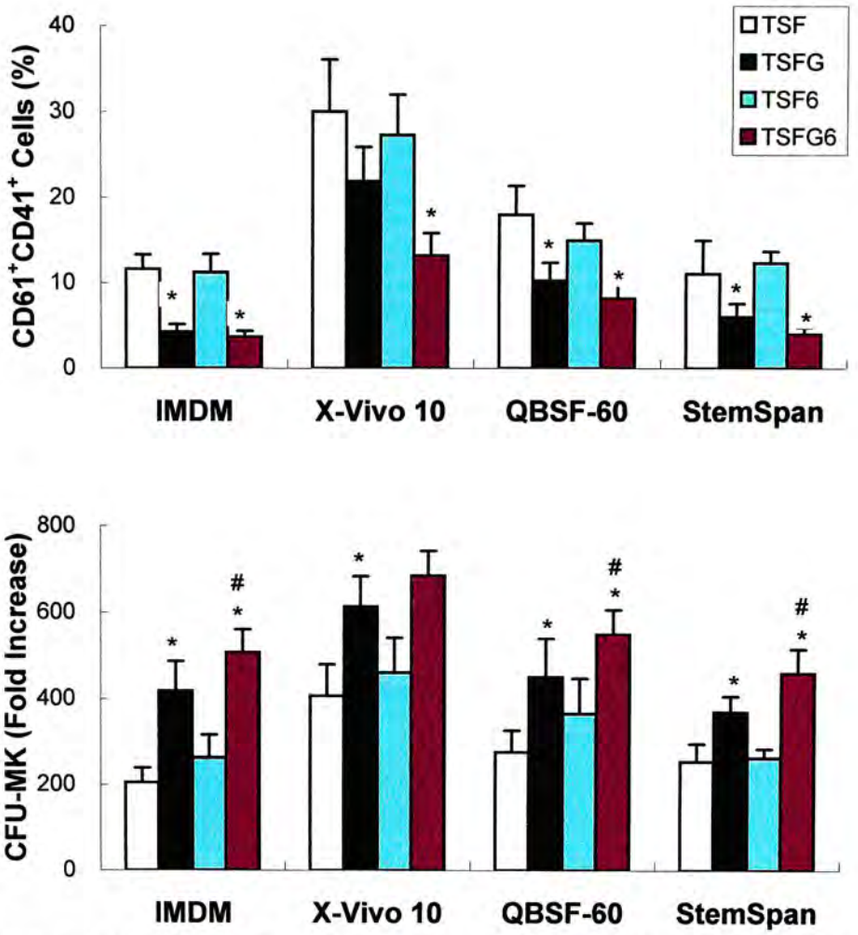


Figure 6.18 Effects of Granulocyte-Colony Stimulating Factor and Interleukin-6 on the Expansion of $CD61^+CD41^+$ Cells and CFU-MK

The expansion of $CD61^+CD41^+$ cells and CFU-MK were analyzed. The percentage of $CD61^+CD41^+$ cells declined and the fold increase of CFU-MK elevated when G-CSF was added to the cultures (* $p < 0.05$). The addition of IL-6 increased the fold increase of CFU-MK ($^{\#}$ $p < 0.05$). $n = 10$

Table 6.6 Effects of Flt-3 Ligand and Increased Dosage of Thrombopoietin and Stem Cell Factor on the *Ex Vivo* Expansion of Cord Blood $CD34^+$ Cells

	TSG50	TSFG50	TSG100	TSFG100
Total Nucleated Cells ($\times 10^6$)	$7.32 \pm 0.55^{\#}$	$12.7 \pm 0.91^{**\#}$	$7.65 \pm 0.74^{\#}$	$13.2 \pm 1.04^{**\#}$
$CD34^+$ Cells (%)	$3.09 \pm 0.49^{\#}$	$7.03 \pm 1.11^{**\#}$	$3.36 \pm 0.55^{\#}$	$8.25 \pm 1.23^{**\#}$
$CD34^+$ Cells ($\times 10^5$)	$2.19 \pm 0.34^{*\#}$	$8.59 \pm 1.53^{**\#}$	$2.88 \pm 0.51^{*\#}$	$10.4 \pm 1.36^{**\#}$
$CD34^+CD38^-$ Cells (%)	$1.32 \pm 0.33^{\#}$	$2.09 \pm 0.48^{**\#}$	$1.72 \pm 0.43^{\#}$	$3.12 \pm 0.73^{**\#}$
$CD34^+CD38^-$ Cells ($\times 10^5$)	$0.96 \pm 0.27^{*\#}$	$2.52 \pm 0.65^{**\#}$	$1.41 \pm 0.36^{*\#}$	$3.85 \pm 0.87^{**\#}$
Total CFU ($\times 10^5$)	$2.65 \pm 0.37^{**\#}$	$4.06 \pm 0.36^{\#}$	$3.12 \pm 0.39^{**\#}$	$4.42 \pm 0.47^{\#}$
CFU-GM ($\times 10^5$)	$1.94 \pm 0.26^{**\#}$	$2.98 \pm 0.26^{\#}$	$2.29 \pm 0.27^{**\#}$	$3.24 \pm 0.33^{\#}$
BFU/CFU-E ($\times 10^4$)	$6.29 \pm 0.97^{**\#}$	$9.57 \pm 0.97^{**\#}$	$10.5 \pm 1.29^{**\#}$	$7.42 \pm 1.05^{**\#}$
CFU-GEMM ($\times 10^4$)	$2.88 \pm 0.36^{**\#}$	$4.51 \pm 0.47^{\#}$	$3.42 \pm 0.42^{**\#}$	$4.93 \pm 0.60^{\#}$
$CD61^+CD41^+$ Cells (%)	$12.7 \pm 1.37^{**\#}$	$8.4 \pm 1.16^{\#}$	$15.6 \pm 1.34^{**\#}$	$8.68 \pm 1.41^{\#}$
$CD61^+CD41^+$ Cells ($\times 10^5$)	$9.35 \pm 1.29^{**}$	10.3 ± 1.62	$13.0 \pm 1.88^{**}$	11.0 ± 1.66
CFU-MK ($\times 10^5$)	$1.06 \pm 0.21^{**\#}$	$1.65 \pm 0.32^{**\#}$	$1.27 \pm 0.24^{**\#}$	$2.76 \pm 0.38^{**\#}$

Enriched $CD34^+$ cells at 2×10^4/ml were cultured for 12 days in QBSF-60 with four cytokine combinations – (1) TSG: 50 ng/ml TPO, 50 ng/ml SCF and 40 ng/ml G-CSF; (2) TSFG: 50 ng/ml TPO, 50 ng/ml SCF, 80 ng/ml FL and 40 ng/ml G-CSF; (3) TSG100: 100 ng/ml TPO, 100 ng/ml SCF and 40 ng/ml G-CSF; (4) TSFG100: 100 ng/ml TPO, 100 ng/ml SCF, 80 ng/ml FL and 40 ng/ml G-CSF. Results were expressed as mean ± S.E. n = 8, * $p < 0.05$ and ** $p < 0.01$ (TSG50 vs TSG100 or TSFG50 vs TSFG100), $^{\#}$ $p < 0.01$ (TSG vs TSFG or TSG100 vs TSFG100).

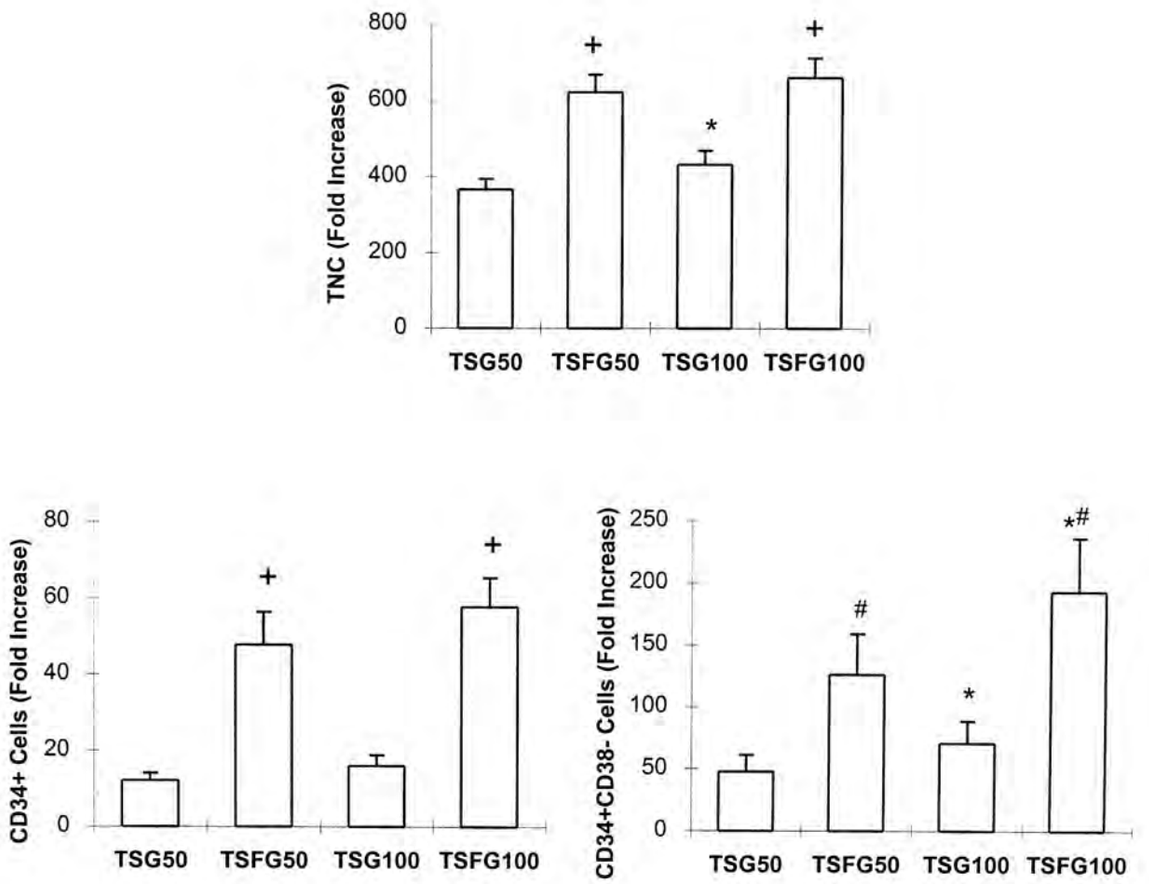


Figure 6.19 Effects of Flt-3 Ligand and Increased Dosage of Thrombopoietin and Stem Cell Factor on the Fold Increase of Total Nucleated Cells, $CD34^+$ Cells and $CD34^+CD38^-$ Cells

Total nucleated cells (TNC), $CD34^+$ cells and $CD34^+CD38^-$ cells were analyzed after 12 days of culture. Increased dosage of TPO and SCF led to higher fold increases of TNC, $CD34^+$ cells and $CD34^+CD38^-$ cells (* $p < 0.05$). The addition of FL also enhanced the expansion of these cell compartments significantly ($^{\#}$ $p < 0.05$ and $^{+}$ $p < 0.01$). n = 8

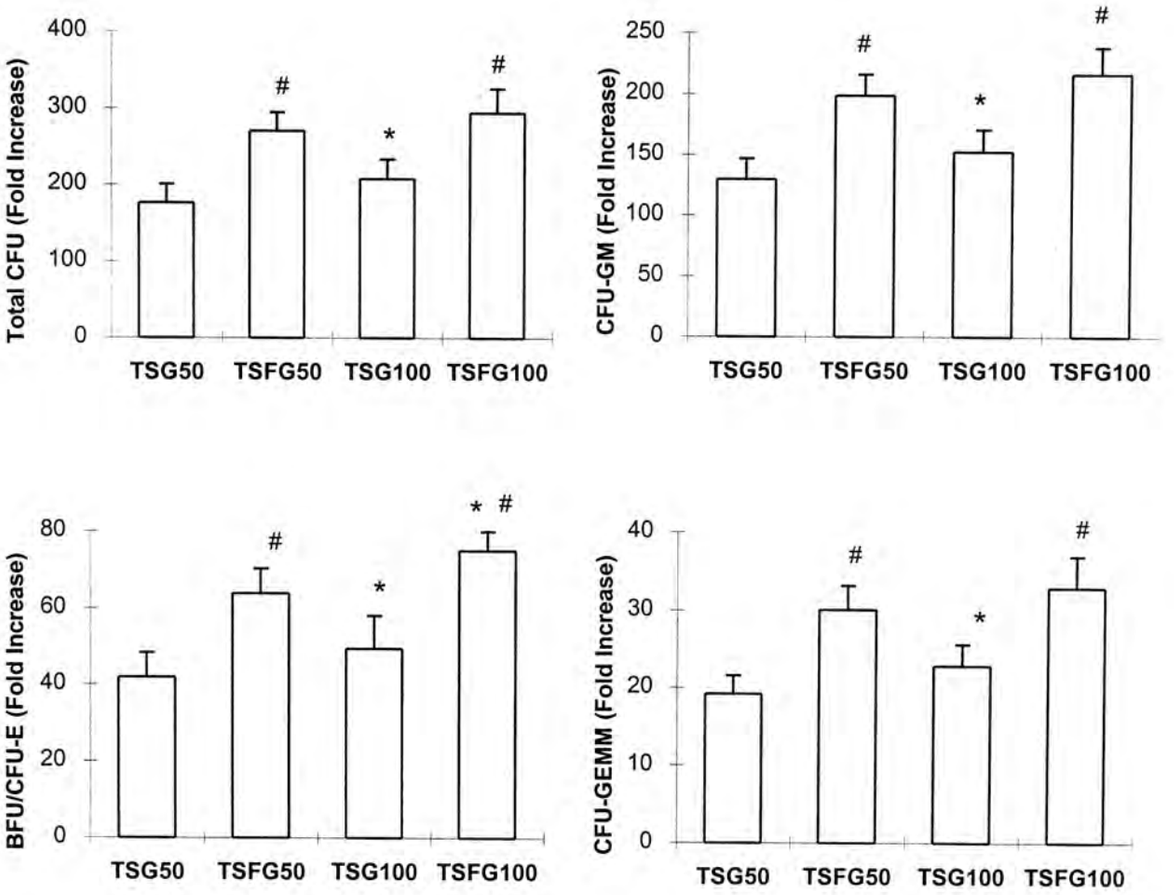


Figure 6.20 Effects of Flt-3 Ligand and Increased Dosage of Thrombopoietin and Stem Cell Factor on the Fold Increase of Different Lineages of CFU

Expanded cells were harvested and assayed for the colony-forming capacity. Increased dosage of TPO and SCF resulted in significant differences on the expansion of CFU (* $p < 0.05$). Significant differences were also observed when FL was added to the cultures ($^{\#} p < 0.01$). $n = 8$

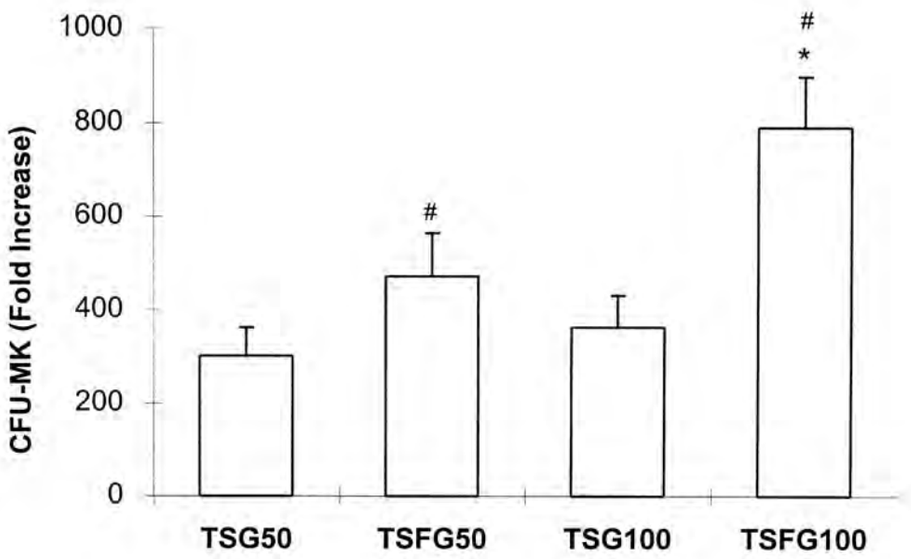


Figure 6.21 Effects of Flt-3 Ligand and Increased Dosage of Thrombopoietin and Stem Cell Factor on the Fold Increase of CFU-MK

Expanded cells were harvested for CFU-MK assay. The addition of FL and increased dosage of TPO and SCF enhanced the expansion of CFU-MK. n = 8, * $p < 0.05$ when comparing 50 ng/ml TPO and SCF with 100 ng/ml TPO and SCF. # $p < 0.01$ when investigating the effects of FL.

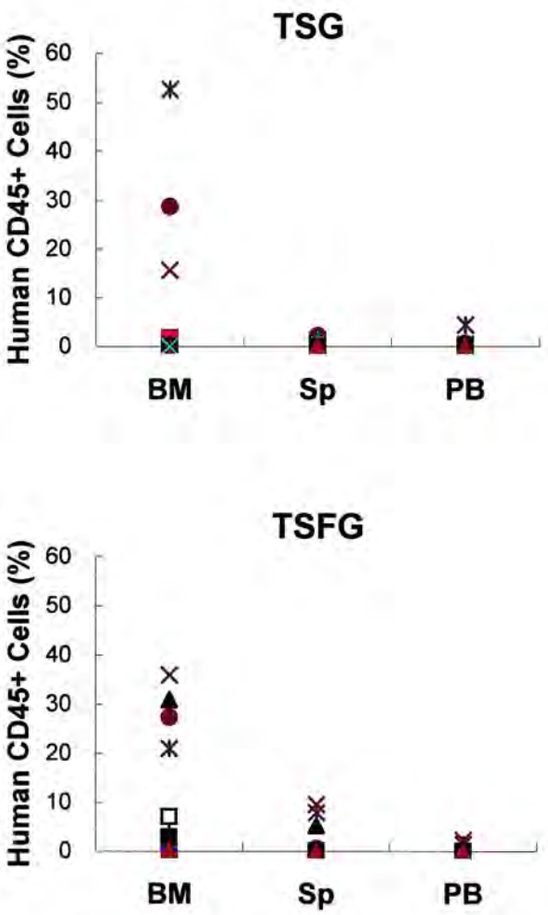


Figure 6.22 Engraftment of Expanded Human Cells in NOD/SCID Mice

Enriched $CD34^+$ cells expanded in QBSF-60 for 12 days with 50 ng/ml TPO, 50 ng/ml SCF and 40 ng/ml G-CSF ± 80 ng/ml FL, were infused into irradiated NOD/SCID mice. After 6 weeks, the engraftment of human $CD45^+$ cells in the bone marrow (BM), spleen (Sp) and peripheral blood (PB) were quantified by flow cytometry. Results were expressed as the percentage of viable cells. n = 13 for TSG and n = 12 for TSFG.

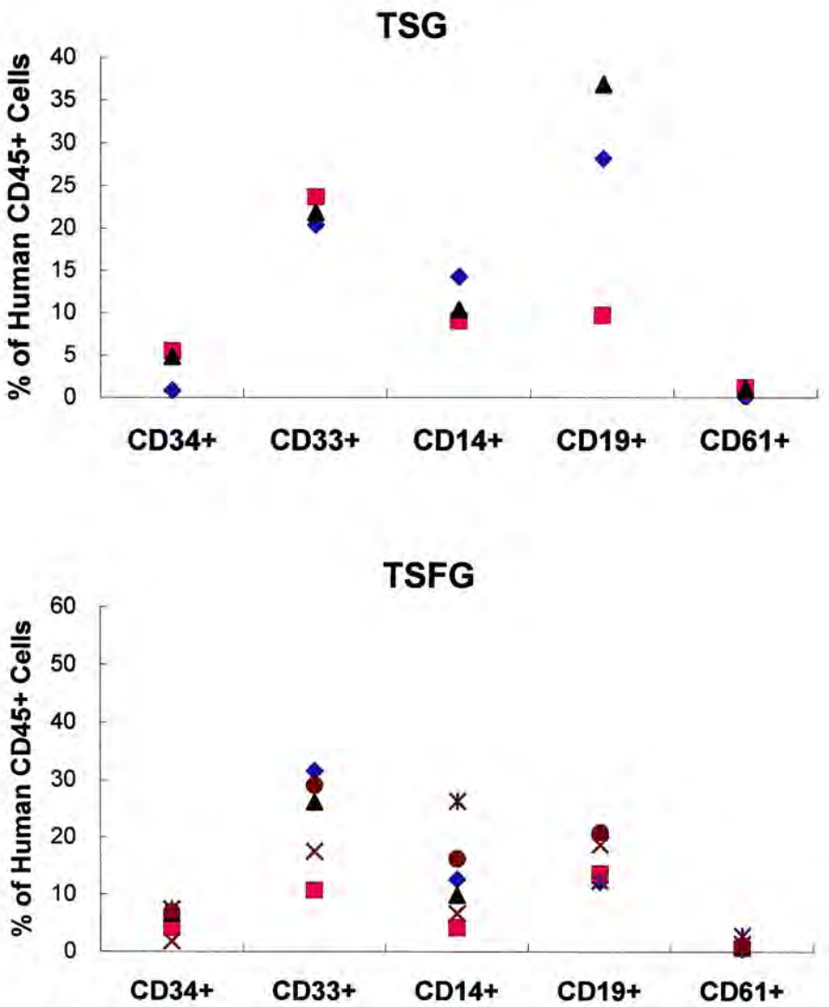


Figure 6.23 Subsets of Human Hematopoietic Cells in the Bone Marrow of Engrafted NOD/SCID Mice

The engraftment of human stem and progenitor ($CD34^+$), myeloid ($CD33^+$, $CD14^+$), lymphoid ($CD19^+$) and megakaryocytic ($CD61^+$) cells were detected in the BM of transplanted NOD/SCID mice after 6 weeks of transplantation. Results were expressed as the percentage of human $CD45^+$ cells.

CHAPTER SEVEN

GENERAL DISCUSSION AND CONCLUSION

The *ex vivo* expansion of CB stem and progenitor cells has been suggested as a strategy of overcoming the insufficient cell dose in a collection. If successful, the expanded cell population might reduce the morbidity, mortality and cost associated with posttransplant neutropenia and thrombocytopenia. In addition, the expanded CB might be suitable for transplanting patients of large body weight. Limited clinical trials have been performed using *ex vivo* expanded CB for transplantation and the outcomes of these studies were inconsistent. At present, no standard protocol has been recommended for the *ex vivo* expansion of CB stem cells.

The aims of this project were to optimize the culture conditions for the *ex vivo* expansion of enriched CB $CD34^+$ cells by investigating the effects of cytokines, culture durations, media, serum supplements and a novel MBL on the system. The read-out assays included $CD34^+$, $CD34^+CD38^-$, $CD61^+CD41^+$ cells, CFU of the GM, E and MK lineages and SRC.

Comparing the effects of two early-acting cytokines FL and SCF on the expansion of the megakaryocytic progenitor cells in the presence of TPO, IL-3 and IL-6, we demonstrated that FL supported more efficient expansion of $CD34^+$ cells and CFU-MK up to 23.8- and 584-fold, respectively. FL receptors were undetectable in megakaryocytic cell lines, suggesting that FL might exert its effects at the stage of stem and progenitor cells. In QBSF-60 serum-free medium and cytokines TPO, SCF and G-CSF, the addition of FL again improved the expansion outcomes of all cell types studied. In this system, the SRC in the spleen were significantly higher in the

NOD/SCID mice infused with FL-expanded cells although only a trend was observed in the BM of these animals.

Our preclinical study demonstrated that QBSF-60 supported the highest yields of early progenitors $CD34^+$ cells, $CD34^+CD38^-$ cells and CFU-GEMM among four media compared. Interestingly, X-Vivo 10 enhanced the highest expansion of megakaryocytic progenitors, $CD61^+CD41^+$ cells and CFU-MK. In addition, the culture duration of 12 days with 100 ng/ml TPO, 100 ng/ml SCF, 80 ng/ml FL and 40 ng/ml G-CSF in the absence of autologous plasma appeared to be the optimal condition for the expansion.

It is of great concern that the pluripotent stem cells might be exhausted during *ex vivo* expansion and for this reason, most clinical trials were performed with the infusion of expanded cells together with unmanipulated cells. In this study, we investigated the effect of a novel MBL on the preservation of early stem cells and demonstrated that MBL at 200 ng/ml significantly preserved CFU-GEMM for 35 days without serum or cytokine supplements. MBL also enhanced the *ex vivo* expansion of $CD34^+CD38^-$ cells and CFU-GEMM in the presence of TPO, SCF and FL. However, the SRC were not expanded, possibly because of the sensitivity of the model. Future studies will be focused on a modified animal model such as the NOD/SCID-$\beta 2m^{-/-}$ mice, which might be able to distinguish the subtle differences between long-term and short-term SRC.

For future studies, we are pursuing to establish the optimized system for the clinical-scale expansion of CB $CD34^+$ cells. We anticipated that similar conditions could be

applied for the expansion of BM and mobilized PBSC. Considering the preferential conditions for the expansion of early/myeloid progenitor cells and the megakaryocytic progenitor cells in terms of culture media and cytokine requirements, we strongly suggested that these two classes of progenitor cells be expanded in separate culture systems.

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